

Four new representatives of the genus *Alloccyclops* Kiefer, 1932 from semi-consolidated subsoil aquifers in Benin (Copepoda, Cyclopoida, Cyclopidae)

Frank Fiers^{1,2}, Moïssou Lagnika³

1 Current address: Royal Belgian Institute of Natural Sciences, Vautierstraat 29, B-1000 Brussels, Belgium

2 “Emil Racoviță” Institute of Speleology, Roman Academy, Clinicilor 5, PO Box 58, Cluj-Napoca, Romania

3 Département de Zoologie, Faculté des Sciences et Techniques, Université d'Abomey-Calavi, 01 BP: 526, République du Bénin

Corresponding author: Frank Fiers (frank.fiers@naturalsciences.be)

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Abstract

Several species of cyclopoid copepods were collected from improved and unimproved hang-dug-wells in the Republic of Bénin over the years 2009–2014. Fifty five wells located in seven different districts were sampled: 15 wells in the district of Pobè (Department Plateau) and 1 well in Kétou (Department Plateau), 4 wells in Porto-Novo District (Department Ouémé) and, 15 wells in Lokossa District (Department Mono), 18 wells in Parakou District (Department Borgou), 1 well in Abomey-Calavi District (Department Atlantique) and 1 in Zogbodomé District (Department Zou). Among them, 4 new species of the genus *Alloccyclops* Kiefer, 1932 were found and are described herein: *Alloccyclops spinifer* sp. n., *A. nudus* sp. n., *A. pilosus* sp. n. and *A. sakitii* sp. n. They are compared with the three African species previously described. *Alloccyclops* appears to be a sub-Saharan taxon specialized to thrive in the variable environment of subsoil aquifers in laterite deck beds. An identification key to the 7 different African *Alloccyclops* species is provided.

Keywords

Copepoda, Cyclopidae, subterranean, Bénin, new species, key, laterites

Introduction

Within the context of a research program in the Republic of Bénin on the biodiversity of the subterranean realm in relation to water quality, pollution vulnerability and local use 48 wells were regularly sampled over a period of one year. Seven wells located in the close vicinity of the monitored wells were occasionally visited. Six of the seven areas are located in the drainage basin of the Ouémé: the area of Parakou in the north of the basin and the area of Kétou, Zogbodomé, Pobè, Abomey-Calavi and Porto-Novo in the south. The seventh study area, the district of Lokossa, belongs to the lower part of the Mono-Couffo drainage basin (see Fig 1). Physico-chemical characteristics of the Pobè and Parakou wells have been published (Lagnika et al. 2014a; 2014b). Analyses of the other regions are currently under study.

The Republic of Bénin, a country 650 km long and 325 km at its widest, in South-West Africa is a sub-Saharan savannah region enduring a tropical climatologic regime: humid in the south, semi-arid in the north (Kottek et al. 2006; Peel et al. 2007). The principal rainy season spans from April to late July. A less intense rainy period is active in the southern part of the country from late September to November. The dry seasons are from December to April and from late July to early September. Wells, dug in the thick laterite deck beds, are of vital importance for the communities during the eight months of drought. They also provide possibilities to study various aspects of this subterranean habitat and the local human impact on this extreme variable environment (Bonsor et al. 2013).

African continental waters have been a very attractive study subject in zooplankton research. Large regions, however, of this vast continent remain unexplored (Dussart 1980). This is particularly true in regards of the fauna thriving in subsoil aquifers for which certain taxonomic groups are ignored because of inadequate sampling and, mainly, the lack of specialists (Boutin et al. 2011; Tuekam Kayo et al. 2012).

Among the copepods collected during this intensive study (i.e. mostly representatives of *Afrocylops* Sars, 1927, *Thermocylops* Kiefer, 1927, *Mesocylops* Sars, 1914) no less than four different species which undoubtedly belong to the cyclopid genus *Alloocylops* were detected. They are described herein and compared with the 3 other African *Alloocylops* previously known.

Material and methods

In the following Tables 1–4 location and well characteristics are summarised (italics refer to the entries in the tables): “Total depth” of the well from ground level; “Ø” diameter of the well at ground level; “Water-level fluctuation”: difference between highest level and lowest level of the water column; “Wall characteristics” differentiate the wells as either a hand-dug well with un-cemented “*natural*” walls, or “*cemented*” i.e. constructed by cemented concrete rings with the lower parts perforated; “Well closure” refers to the protection from superficial influences: wells can left unprotected and are “*open*” or closed by a pave “*slab*”, corrugated “*iron*” or “*wood*”; “Principal use”: “*potable*” means exclusively used as drinking

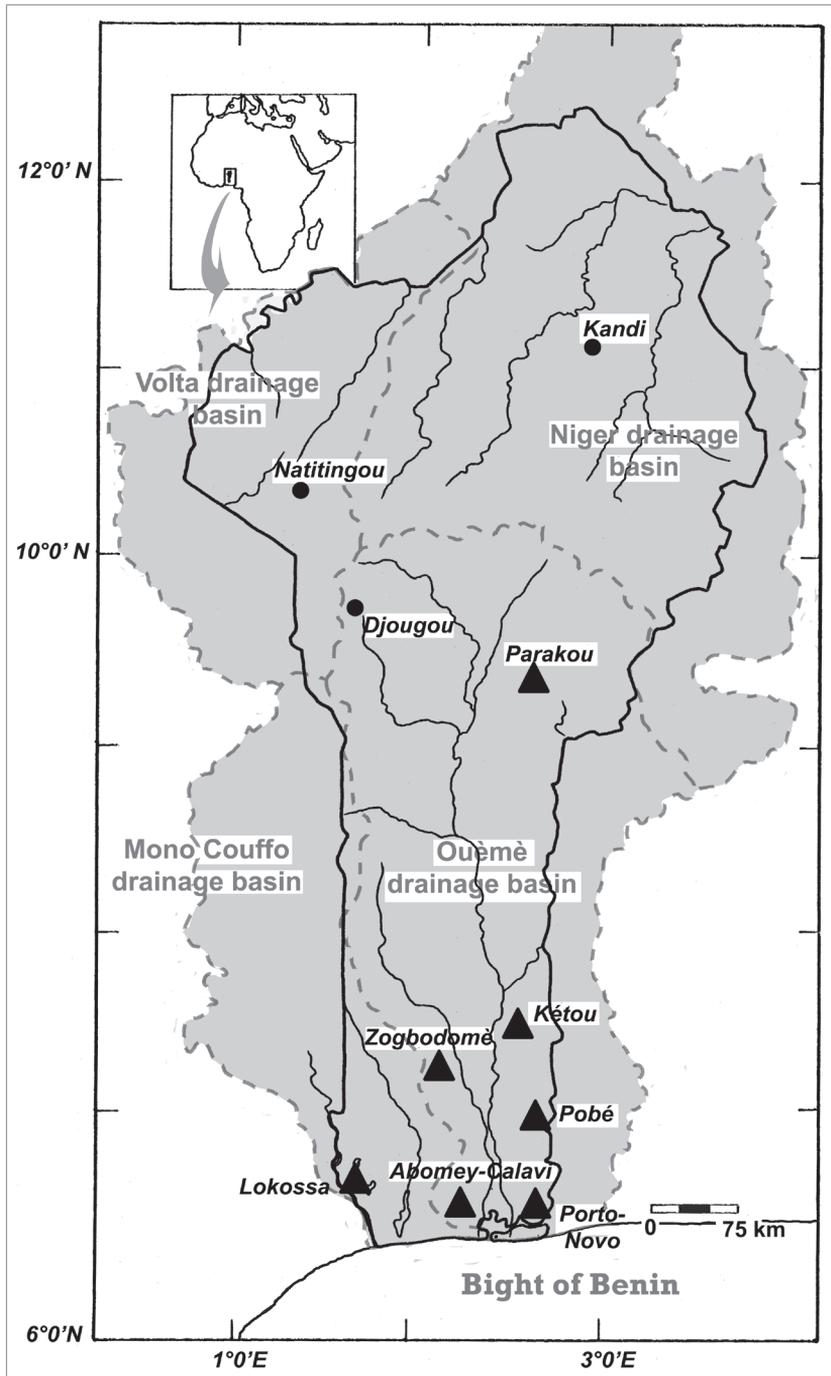


Figure 1. Map of Bénin indicating the study areas (black triangles): Lokossa stands for 15 sampled wells, Porto Novo for 4 sampled wells, Pobé for 15 sampled wells and Parakou for 18 sampled wells. Grey dashed lines contour the limits of the 4 different drainage basins, from http://eaubenin.bj/docs/Bassins_Versants/Atlas_Hydrographique_Rapport_final.pdf (accessed November 2014).

Table 1. Sample localities and principal well characteristics in the district of Lokossa: presence of *Alloyclops* species (dark grey background), other copepods (light grey background) and absence of Cyclopoida (white background).

Station	Total depth (m)	Ø (m)	Water level fluct. (m)	Wall character.	Well closure	Principal use	Location
P1	31,90	2,00	1,25	cemented	slab	potable	06°42'29.5"N, 01°48'11.8"E
P2	41,30	1,45	3,00	cemented	slab	potable	06°42'29.2"N, 01°48'11.9"E
P3	46,15	2,00	4,80	cemented	slab	potable	06°45'24.9"N, 01°49'11.7"E
P4	27,75	2,00	5,00	cemented	slab	potable	06°42'41.0"N, 01°44'46.7"E
P5	14,10	1,20	6,30	natural	open	potable	06°43'12.5"N, 01°44'02.7"E
P6	13,15	1,10	3,70	natural	open	potable	06°43'24.8"N, 01°44'39.2"E
P7	4,45	1,00	2,00	cemented	iron	domestic	06°38'22.8"N, 01°42'54.7"E
P8	5,00	1,45	0,50	cemented	slab	domestic	06°39'01.3"N, 01°40'32.1"E
P9	5,60	0,90	1,20	cemented	open	domestic	06°38'43.2"N, 01°42'26.8"E
P10	11,65	2,10	2,90	cemented	slab	domestic	06°40'03.5"N, 01°47'43.4"E
P11	10,70	0,90	2,10	natural	open	domestic	06°38'54.6"N, 01°46'47.6"E
P12	4,80	1,40	3,70	cemented	open	domestic	06°38'14.8"N, 01°46'54.3"E
P13	5,70	1,20	2,60	cemented	slab	domestic	06°41'29.0"N, 01°39'02.2"E
P14	8,85	2,00	6,35	cemented	slab	domestic	06°42'50.1"N, 01°38'17.8"E
P15	2,80	1,30	0,50	natural	open	domestic	06°43'04.1"N, 01°40'59.8"E

Table 2. Sample localities and principal well characteristics in the district of Parakou: presence of *Alloyclops* species (dark grey background), other copepods (light grey background) and absence of Cyclopoida (white background).

Station	Total depth (m)	Ø (m)	Water level fluct. (m)	Wall character.	Closure	Principal use	Location
A1	7,78	1,2	1,18	cemented	iron	potable	09°16'22.7"N, 02°34'54.7"E
A2	6,95	1,05	0,85	cemented	open	domestic	09°19'40.3"N, 02°37'28.8"E
A3	5,9	1,25	1,15	natural	iron	domestic	09°20'53.9"N, 02°36'44.0"E
A4	13,15	1,5	4,35	cemented	iron	potable	09°18'42.1"N, 02°36'07.6"E
A5	8,4	1,2	3,1	natural	open	domestic	09°20'51.8"N, 02°36'14.8"E
A6	8,85	1,2	0,2	cemented	open	domestic	09°20'18.7"N, 02°37'36.3"E
B1	7,9	0,9	3,5	cemented	iron	potable	09°19'57.2"N, 02°39'40.6"E
B2	6,6	1,45	0,8	cemented	iron	potable	09°19'55.2"N, 02°39'40.5"E
B3	7,5	1,4	1,6	natural	slab	domestic	09°20'03.5"N, 02°37'47.2"E
B4	8,0	1,2	3,1	cemented	open	domestic	09°20'08.1"N, 02°37'45.4"E
B5	10,45	1,15	5,95	natural	open	domestic	09°20'18.0"N, 02°37'51.1"E
B6	16,15	1,4	10,55	cemented	iron	gardening	09°21'33.4"N, 02°38'32.4"E
C1	8,8	1,1	2,1	cemented	iron	domestic	09°23'28.7"N, 02°37'21.4"E
C2	14,2	1,8	5,5	cemented	iron	potable	09°23'05.5"N, 02°37'23.2"E
C3	10,8	1,2	5,8	cemented	iron	potable	09°21'58.4"N, 02°37'30.6"E
C4	5,5	1	0,9	natural	open	domestic	09°21'45.4"N, 02°37'46.6"E
C5	12,85	2	7,25	cemented	iron	potable	09°21'56.9"N, 02°36'41.2"E
C6	11,2	1,1	5,3	cemented	slab	potable	09°21'01.7"N, 02°37'37.6"E

Table 3. Sample localities and principal well characteristics in the district of Pobè: presence of *Alloccyclops* species (dark grey background), other copepods (light grey background) and absence of Cyclopoida (white background).

Station	Total depth (m)	Ø (m)	Water level fluct. (m)	Wall character.	Well closure	Principal use	Location
Pb1	17,30	1,40	2,00	natural	open	potable	06°58'57.3"N, 02°40'32.4"E
Pb2	36,08	1,40	3,70	cemented	slab	potable	06°58'57.1"N, 02°39'54.9"E
Pb3	40,80	1,40	3,60	cemented	slab	potable	06°58'49.7"N, 02°39'19.6"E
Pb4	50,50	1,45	3,20	cemented	slab	potable	06°57'48.1"N, 02°38'50.2"E
Pb5	16,20	1,50	2,90	cemented	slab	potable	06°59'13.6"N, 02°39'56.6"E
Pb6	19,60	1,05	17,45	cemented	wood	domestic	07°12'03.5"N, 02°36'04.4"E
Pb7	6,10	0,60	3,75	natural	iron	domestic	07°09'28.7"N, 02°39'09.2"E
Pb8	26,27	1,50	7,30	cemented	open	domestic	07°02'10.1"N, 02°42'16.7"E
Pb9	19,25	1,40	4,65	cemented	wood	potable	07°09'23.5"N, 02°44'09.5"E
Pb10	17,60	1,60	5,75	cemented	open	potable	07°09'18.2"N, 02°44'10.6"E
Pb11	13,90	1,10	7,45	natural	iron	domestic	07°08'59.4"N, 02°45'02.1"E
Pb12	14,90	1,25	1,20	cemented	slab	potable	06°58'05.0"N, 02°41'47.9"E
Pb13	34,52	1,55	4,50	cemented	slab	potable	06°57'48.4"N, 02°42'10.1"E
Pb14	36,00	1,52	2,20	cemented	slab	potable	06°57'26.0"N, 02°42'25.9"E
Pb15	31,00	1,20	2,70	cemented	open	potable	06°57'39.6"N, 02°42'14.4"E

Table 4. Sample localities and principal well characteristics in the district of Porto-Novu: presence of *Alloccyclops* species (dark grey background).

Station	Total depth (m)	Ø (m)	Water level fluct. (m)	Wall character.	Closure	Principal use	Location
AR	7,80	0,90	3,00	cemented	iron	domestic	06°29'04.2"N, 02°38'24.3"E
SA	7,00	0,90	2,00	natural	open	domestic	06°29'02.4"N, 02°38'26.0"E
LB	10,50	0,85	2,85	natural	open	domestic	06°29'05.7"N, 02°38'25.3"E
AH	4,00	1,00	2,50	natural	iron	domestic	06°29'18.8"N, 02°38'14.8"E

water, “domestic” when the water is used for all purposes, and “gardening” when used only for private household gardens. Sample dates are provided with the description of each species. Detailed information on the different drainage basins was extracted from the reports made available through the “*Direction Générale de l’Eau*” (2008).

The occasionally sampled wells: at Abomey-Calavi located adjacent to the university grounds in the village of Zogbadjè (11.75 m deep, 1 m Ø, 2.10 mean water level, natural walls, left open, domestic purposes; well location: 6°25'32.2"N, 002°20'17.2"E; Department Atlantique); village well at Kéwi in the district of Kétou (open, natural, abandoned well in secondary forest; district centre: 7°21'29"N, 2°36'27"E; Department Plateau) and well at Zogbodomé situated close to the main road (open natural domestic use; district centre 7°05'N, 2°06'E; Department Zou).

Biological samples of the water column were taken with a Cvetkov plankton net (funnel 200 µm mesh size, 150 µm below valve). Baited traps were used to collect bottom dwelling animals. Total depth, diameter (Ø), and mean water depth were measured with a decametre and the exact location with a Garmin® GPS60. Samples were fixed at the spot adding 100 ml of a 7% formaldehyde solution. Sorted out at the laboratory of the Zoology Department of the Abomey-Calavi University, each taxon has been stored separately and preserved in 70% ethanol.

Copepods were first observed in temporary slides (mounted in glycerol). Dissected animal parts are mounted on permanent slides in glycerol, the cover glasses sealed with a polyurethane varnish. Preserved specimens are stored in 75% buffered ethyl alcohol (with 5–10% glycerol). All material is stored in the Crustacea collection of the Royal Belgian Institute of Natural Sciences and inventoried with acc. nr RBINSc COP #. Observations and illustrations were made on a Leitz Diaplan phase contrast microscope equipped with a camera lucida. Abbreviations used in the descriptions: Aesth, aesthetasc; CIV-CV, copepodid stages 4 and 5; EXO, END, exopodite and endopodite, respectively; L/W ratio, length/width ratio; armature distribution of the leg ramal segments: Roman numerals refer to spines, Greek numerals to setae.

Taxonomy

Family Cyclopidae Rafinesque, 1815

Genus *Allocyclops* Kiefer, 1932 *amend.* Fiers, 2012

Allocyclops spinifer sp. n.

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Figs 2, 3, 4A, B, I, 5A–C, 6

Type locality. Ishèko (Pobè District, Ouémé drainage basin), Well Pb5: West from Pobè center along the principal road to Kétou District (details in Table 3).

Type material. Female holotype, dissected and mounted on 3 slides (RBINSc COP 10.304A-C), allotype male, dissected and mounted on 3 slides (RBINSc COP 10.305A-C), 1 female paratype dissected on 2 slides (RBINSc COP 10.306A-B) and 4 females, 5 males and 4 juveniles preserved (RBINSc COP 10.307), all collected 26/07/2012; additional paratypic material: 3 females, 4 males, 5 juveniles (preserved material RBINSc COP 10.353), topotypic, collected 16/04/2013.

Etymology. derived from the Latin words *spinula* and *ferre*, respectively meaning small thorn and to carry, and referring to the spinular ornamentation on pediger 5, on leg 4 and on the caudal rami.

Additional material.

(1) Pobè District, Ouémé drainage basin:

- Well Pb6: 07/02/11: 6 ♀♀, 2 ♂♂ and 2 CV copepodids (♀ dissected RBINSc COP 10.308A-C, preserved material RBINSc COP 10.309); 27/11/2012:

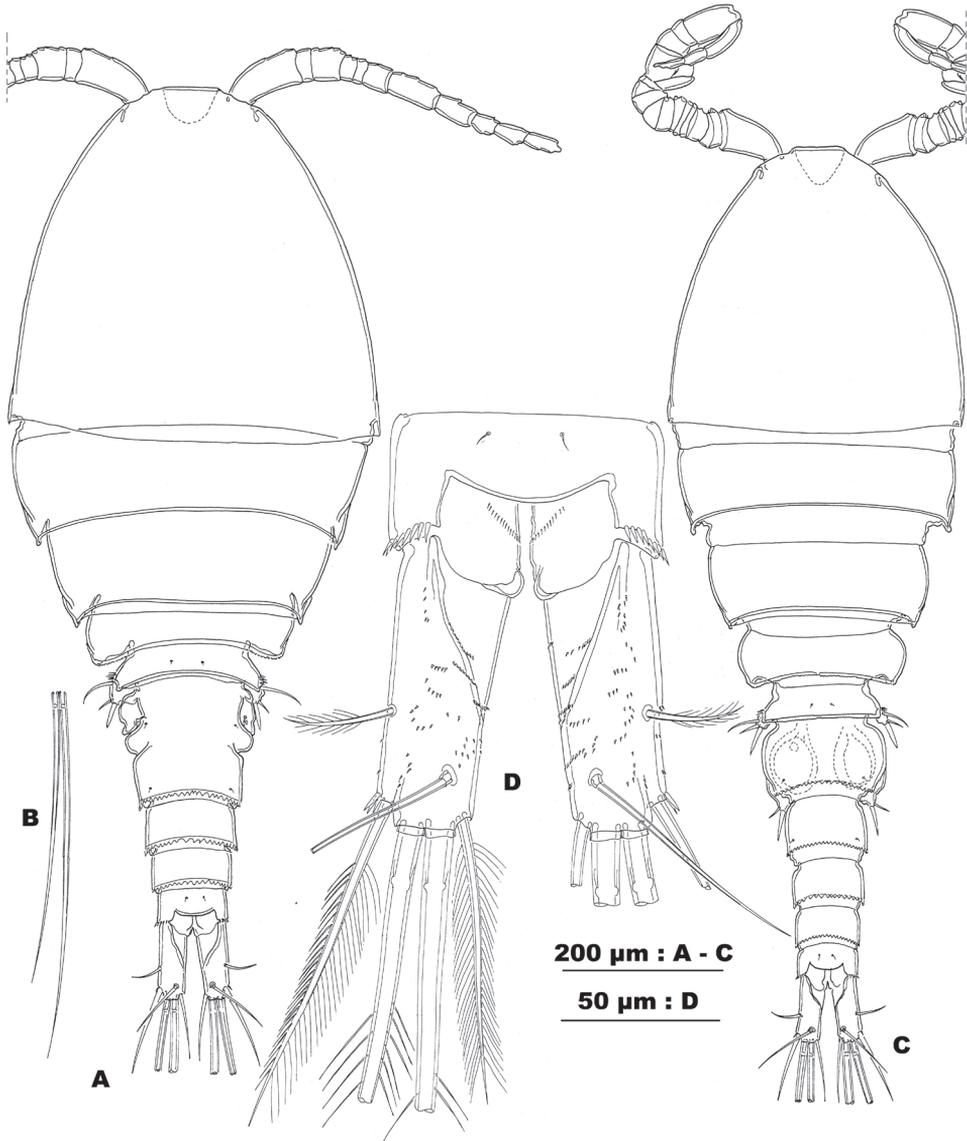


Figure 2. *Alloocylops spinifer* sp. n. **A** habitus in dorsal view **B** principal setae of left caudal ramus **C** habitus in dorsal view **D** anal somite and caudal rami, dorsal view. (Female RBINSc COP 10.304: **A**, **B**, **D**; male RBINSc COP 10.305: **C**).

19 ♀♀, 5 ♂♂, 1 CIV & 1 CV ♀ (CV dissected RBINSc COP 10.386A-B, preserved material RBINSc COP 10.327); 04/03/2013: 21 ♀♀, 11 ♂♂, 5 juveniles (preserved material RBINSc COP 10.339); 26/11/2012: 2 ♀♀ (preserved material RBINSc COP 10.377); 20/09/2013: 9 ♀♀, 2 ♂♂ (preserved material RBINSc COP 10.376).

- Well Pb12: 24/07/2012: 3 ♀♀, damaged (preserved material RBINSc COP 10.328).

(2) Zogbodomè District, Ouémé drainage basin:

- Well Zogbodomè: 21/10/2009: 2 ♀♀, 1 ♂ (preserved material RBINSc COP 10.387).

Description. *Female.* Habitus (Fig. 2A) typically cyclopid with prominent prosome, 1.6 times longer than urosome. Body length 1175 µm (holotype), ranging from 1075 to 1200 µm (n=5). Urosome rather narrow, compared to wide cephalothorax and pediger 2. Urosomite 1 (pediger 5) with cluster of long and rigid spinules on the lateral and ventrolateral surface (Fig. 3D). Genital double-somite widest in anterior third, with distinct constriction of lateral margins in anterior half and a L/W ratio: 1/1.25. Ventral surface with, in frontal third, a transverse undulate ridge parallel with lateral arms of genital complex (dotted line between arrows in Fig. 3A) and, caudally from copulatory pore, some crescent ridges. Posterodorsal fringe of prosomites 1–3 narrow and un-incised, of prosomite 4 and first urosomite narrow and discretely serrate. Posterior margin of genital double-somite and urosomites 4–5 wide and serrate, coarsely incised. Anal somite with smooth crescent operculum and large anal sinus. The latter with an oblique row of minute and slender spinules on either side of the anal slit. Posterior margin of anal somite with uninterrupted row of spinules. Dorsal and lateral surface of urosomites adorned with minute, hardly visible spinules. Ventral surface of anal somite with an irregular pattern of slightly larger spinules.

Caudal rami (Fig. 2D) cylindrical, 2.7–3.0 times longer than wide, with distinct triangular depression in anterior half of medial margin. Anterolateral seta short, pinnate, and inserted in posterior half, without spinules at insertion. Distolateral element slightly longer than ramus (ratio 1.2–1.3/1) and longer than medial terminal element (ratio 1.2–1.4/1). Distolateral element with widely spaced long and slender spinules along outer side of stem, with widely spaced setules along inner side; spinules present near its insertion. Medial element as long as caudal ramus, and pinnate. Dorsal seta, articulating on small basal part, shorter than ramus (ratio 0.8–0.9/1). Principal terminal caudal setae pinnate, with breaking plane present in both. Surface of rami with irregular and defuse pattern of minute spinules on dorsal and ventral side, triangular anteromedial depression clearly delimited dorsally.

Antennule 11-segmented, not reaching beyond cephalothorax, and having the typical armature distribution *i.e.*: 1(8)-2(4)-3(6)-4(2)-5(2)-6(2)-7(3)-8(2+Aesth.)-9(2)-10(2+Aesth.)-11(7+Aesth). First segment with crescent row of slender spinules in proximal half (Fig. 4A). Aesthetasc on segment 8 half as long as accompanying seta, linguiform, and reaching just beyond distal margin of segment 8. Aesthetasc on segment 10 filiform, shorter than accompanying element and extending to middle of terminal antennular segment. Terminal aesthetasc tubular, slightly longer than segments 10–11 combined, and fused at base with seta; both equally long (Fig. 4B).

Antennal general aspect and armature as in *A. cavicola*: *i.e.*: 4-segmented, short exopodal seta, 2 abexopodal setae, first endopodite segment with 1, second one with 9, and ultimate segment with 7 elements. Frontal surface of coxobasis (Fig. 5A) with 2

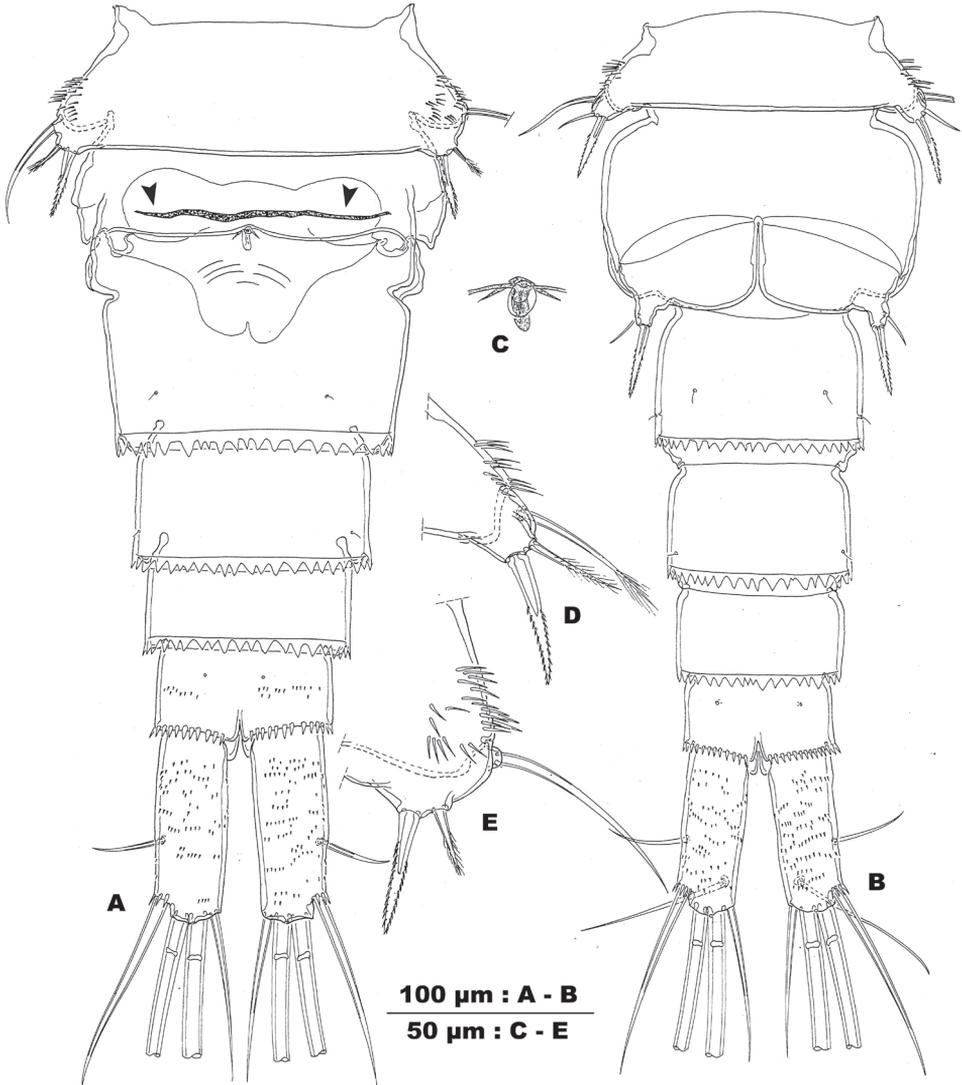


Figure 3. *Alloctyclops spinifer* sp. n. **A** urosome in ventral view **B** urosome in ventral view **C** copulatory pore and duct **D** leg 5 and lateral part of pediger, in ventral view **E** leg 5 and lateral side of pediger in ventral view. (Female RBINSc COP 10.304: **A, C**; RBINSc COP 10.306: **E**; male RBINSc COP 10.305: **B, D**).

rows of coarse spinules parallel to outer margin (proximal one with 10–12, distal one with 9–10 spinules) and short proximal transverse row of tiny spinules near abexopodal margin. Caudal surface (Fig. 5B, C) with 3 clusters of long and slender spinules in proximal half: 2 clusters near outer margin, 1 near inner margin.

Mandible, maxillule and maxilliped as in *A. cavicola*. General aspect of maxilla as in *A. cavicola*. Medial claw of maxillary basis (Fig. 4I) long and blunt, armed with 4 large teeth increasing in length medially. Accessory seta as long as claw, serrate on both

sides in proximal half, on one side in distal half. Both elements on first endopodite segment with set of large spinules in middle. Distal half either smooth or with minute spinules along both sides of the stem. Terminal element on second endopodite segment articulating on segment, furnished with minute spinules along one side of the stem. Both lateral elements on second segment slender and naked.

Legs 1–4 protopodite (Fig. 6A, C, D, F, G). Praecoxa of legs 1–3 with short row of slender spinules along distal margin close to outer edge. Praecoxa of leg 4 smooth. Outer part of coxa with 2 (legs 1–3) or 3 (leg 4) short rows of spinules on frontal surface. Coxal caudal surface of legs 1–3 with set of spinules near proximal outer edge (see ex. for leg 2: Fig. 6D). Medial coxal seta of legs 1–4 pinnate and reaching beyond proximal endopodite segment. Intercoxal sclerites unadorned on both sides. Lateral distal edges crescent without ornamentation. Mediodistal region concave (legs 1–3: Fig. 6B) or nearly straight (leg 4). Inner edge of leg 1 basis crescent, of legs 2–4 produced in sharp triangular process. Medial margin hairy in legs 1–3, naked in leg 4. Inner element on leg 1 basis present, serrate and reaching beyond proximal endopodite segment, but not beyond middle of distal segment. Outer element on basis of the legs setiform: longest on leg 1 basis, shortest one on basis of leg 2.

Legs 1–4 with 2-segmented rami (Fig. 6A, C, F, G). Exopodite and endopodite equally long. Rami with the following armament distribution:

	EXO	END
Leg 1	I,1 - III,2,3	0,1 - 1,I+1,3
Leg 2	I,1 - III,I+1,4	0,1 - 1,I+1,4
Leg 3	I,1 - III,I+1,4	0,1 - 1,I+1,4
Leg 4	I,0 - II,I+1,4	0,1 - 1,II,3

Outer distal edge of second endopodite segment of legs 1–3 distinctly protruded, of leg 4 small and as large as inner one. Frontal and caudal surface of leg segments of legs 1–2 and frontal surface of leg 3 unadorned. Caudal surface of distal endopodite segment of leg 3, and frontal and caudal surface of leg 4 segments with diffuse pattern of tiny spinules.

Caudal surface of proximal endopodite segment of leg 4 inflated. Distal endopodite segment of leg 4 twice as long as wide with inner terminal spine as long as segment. Outer terminal spine slightly longer than half inner one (ratio: 0.55–0.56). Inner terminal spine set with long slender spinules. Outer subdistal and inner setae, equally long, not reaching noticeably beyond longest (outer) terminal spine.

Leg 5 (Fig. 3E) confluent with pediger, represented by narrow crescent expansion. Ancestral basal segment represented by long pinnate element, and ancestral distal segment marked by narrow truncate elevation, bearing 2 elements: setiform outer one, spiniform medial one, the latter twice as long as the former.

Leg 6 vestiges located laterally with 3 elements: dorsal one short and setiform, middle and ventral one dwarfed, blunt triangular, with hyaline appearance. Valves unadorned. Copulatory pore located midventrally in anterior third of somite, leading to U-shaped duct (Fig. 3C). Anterior receptacle expanded with undulate frontal margin. Posterior receptacle produced caudally extending to middle of double-somite,

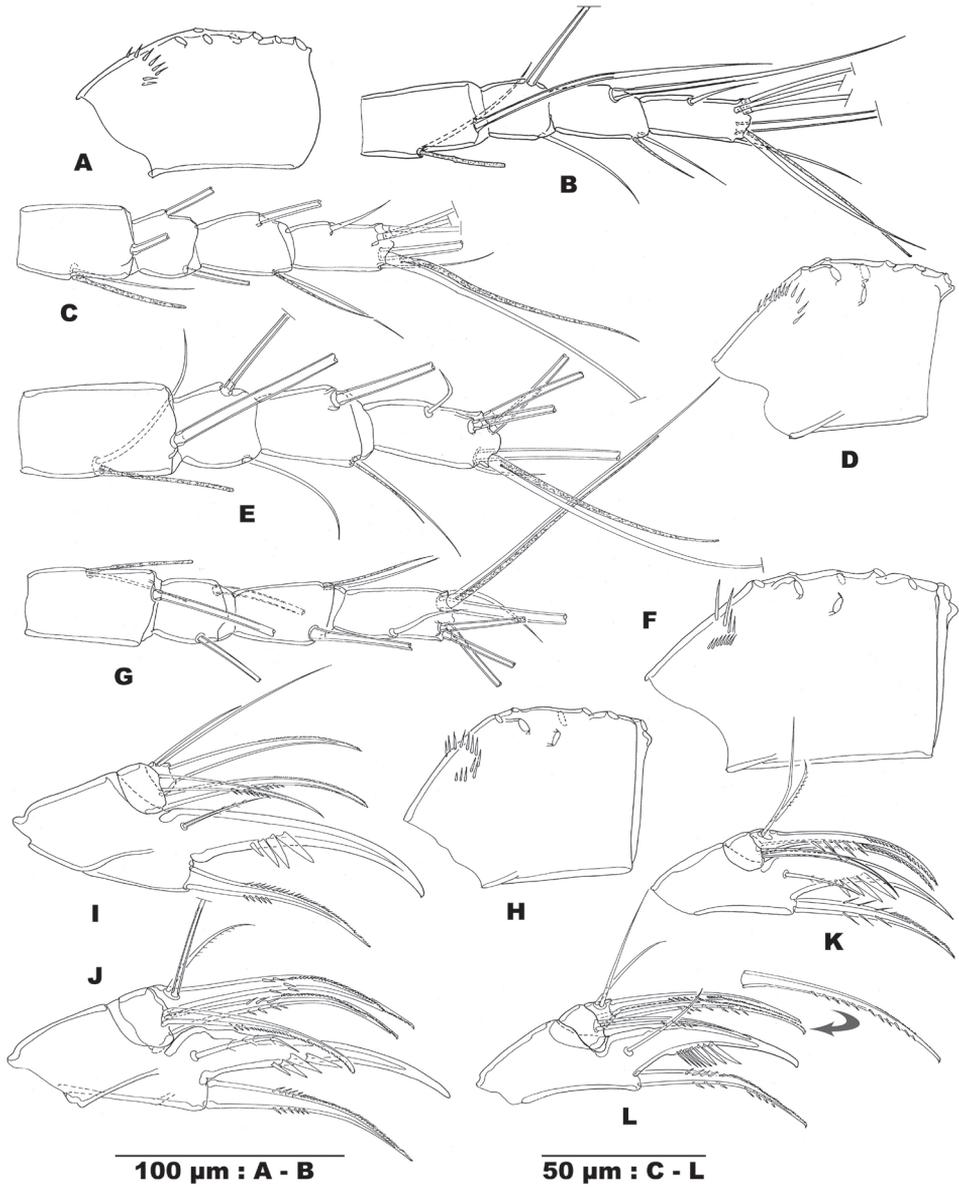


Figure 4. *Alloccyclops spinifer* sp. n. **A** antennary segment 1 **B** antennary segments 8–11; *A. pilosus* sp. n. **C** antennary segments 8–11 **D** antennary segment 1; *A. nudus* sp. n. **E** antennary segments 8–11 **F** antennary segment 1; *A. sakitii* sp. n. **G** antennary segments 8–11 **H** antennary segment 1; *A. spinifer* sp. n. **I** maxillary basis and endopodite; *A. nudus* sp. n. **J** maxillary basis and endopodite; *A. pilosus* sp. n. **K** maxillary basis and endopodite; *A. sakitii* sp. n. **L** maxillary basis and endopodite (RBINSc COP 10.304: **A, B, I**; COP 10.310: **C, D, K**; COP 10.367: **E, D, J**; COP 10.253: **G, H, L**).

bilobed. Lateral arms (= narrow canal-like rim extending from copulatory duct to genital aperture) straight.

Male. Habitus (Fig. 2C) as in female, but with narrower prosome and 6-segmented urosome. Body length: 1000 μm (allotype), ranging from 807–1000 μm (n=5). Integumental ornamentation, fringe structure and L/W ratio of caudal rami as in the female (2.7–3.0/1). Armature: distolateral element 1.27–1.30 times longer than ramus, terminal medial element nearly 0.9–1.0 times ramal length, dorsal seta as long as ramus.

Antennule as described for *A. cavicola*: i.e. 16-segmented with the following armature distribution: 1(8+3 Aesth.)-2(4)-3(2)-4(1)-5(2)-6(2)-7(2)-8(2)-9(2+Aesth.)-10(2)-11(2)-12(2)-13(1+Aesth.)-14(4)-15(3)-16(11+Aesth.). Aesthetascs on segment 1, 9 and 13 long, narrow and linguiform. Mouthparts as in the female. Legs 1–4 with general structure and armature as in the female, but terminal segment of endopodites slightly narrower (see ex. for leg 3: Fig. 6H) and leg 4 proximal endopodite segment not expanded caudally.

Leg 5 (Fig. 3D) with expansion representing distal segment more accentuated than in female. Proportional lengths of armament as in the female. Spinule ornamentation on lateral and ventral surface of pediger less dense than in the female.

Leg 6 (Fig. 3B) represented as a large, unadorned, valve with outer distal edge distinctly produced bearing 2 elements: outer one setiform, half as long as medial spiniform element.

Variability. Animals from Well Pb 6 collected on 07/02/2011 are smaller with the body length of the females ranging from 875 to 1009 μm , that of the males between 890–895 μm . They display shorter caudal rami (L/W ratio 1/2.6–2.7) with slightly longer setae: distolateral one 1.3–1.4 times longer than the ramus, and medial terminal element 1.1–1.3 times longer than ramus. In all other respects they resemble *A. spinifer* sp. n. as described above.

***Allocyclops pilosus* sp. n.**

<http://zoobank.org/FA418D62-E64F-417A-A11E-8B5D3B147922>

Figs 4C, D, K, 5D–F, 7, 8, 9

Syn. *Allocyclops* sp. [*partim*] in Lagnika et al. 2014b

Type locality. Guéma, Parakou District, Ouémé drainage basin: Well C2 (details in Table 2).

Type material. Female holotype, dissected and mounted on 3 slides (RNBNSc COP 10.313A-C), allotype male, dissected and mounted on 2 slides (RBINSc COP 10.312A-B), paratypes: 11 females and 2 males preserved in alcohol (RBINSc COP 10.314), all collected 07/03/2013; and additional (topotypic) paratypes collected on 13/02/2011: 4 females, 1 male, 1 juvenile; on 08/03/2013: 37 females, 15 males, 7 juveniles (preserved material RBINSc COP 10.340).

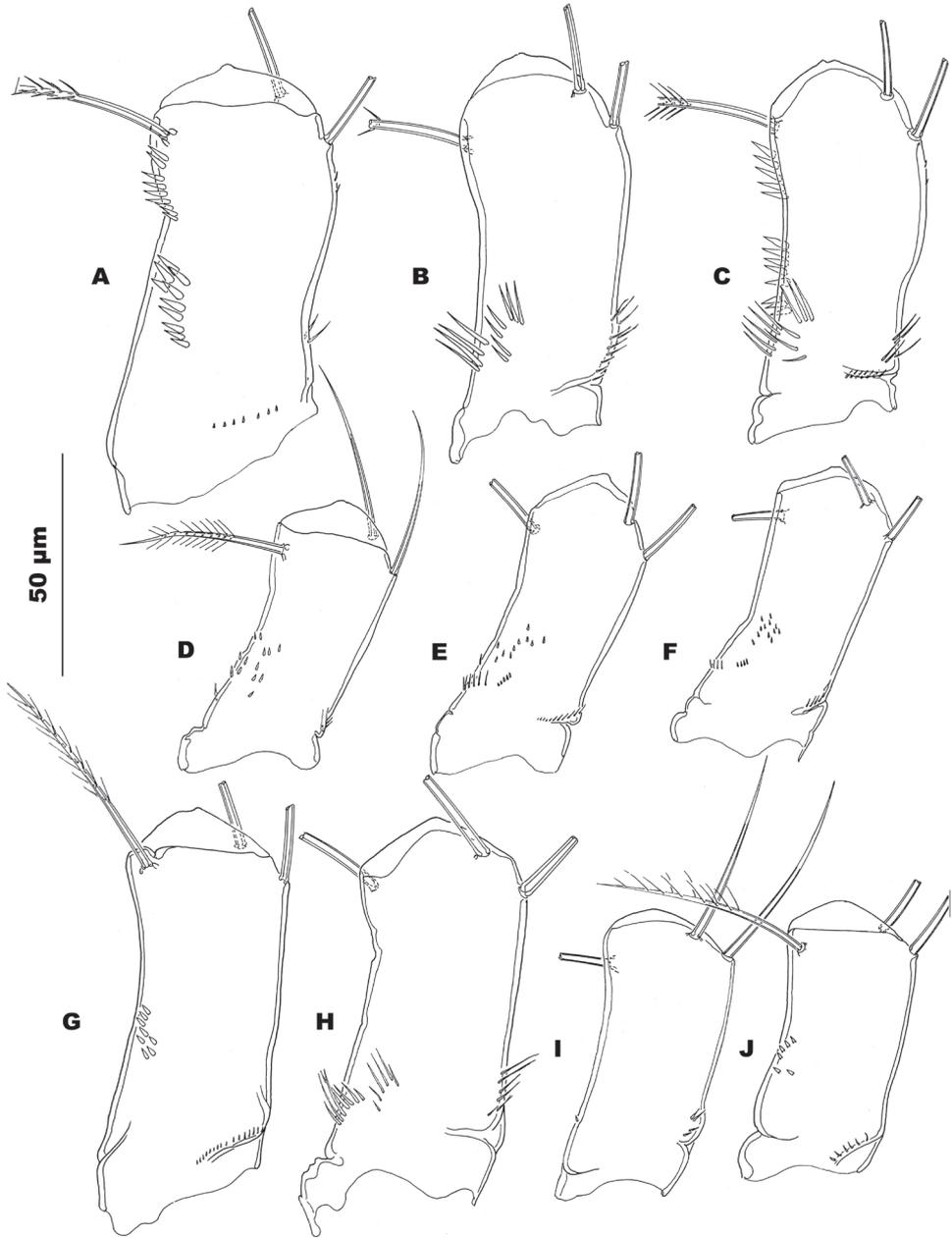


Figure 5. Antennary coxobasis: *Alloctylops spinifer* sp. n. **A** frontal view **B** caudal view **C** caudal view; *Alloctylops pilosus* sp. n. **D** frontal view **E** caudal view **F** caudal view; *Alloctylops nudus* sp. n. **G** frontal view **H** caudal view; *Alloctylops sakitii* sp. n. **I** caudal view **J** frontal view (RBINSc COP 10.304: **A**; COP 10.306B, COP 10.305: **C**; COP 10.310: **D, E**; COP 10.312: **F**; COP 10.3334, COP 10.367; COP 10.355: **I, J**).

Etymology. From the Latin word “*pilus*” meaning hair and refers to the rows of delicate hairs along the medial margin of the caudal rami.

Additional material.

- (1) Parakou District, Ouémé drainage basin:
 - Well A1: 10/07/12: 5 ♀♀, 1 CV juvenile ♀ (preserved material RBINSc COP 10.321);
 - Well B6: 26-10-11: 1 ♀ (preserved material RBINSc COP 10.320); RBINSc COP 10.311);
 - Well C6: 13/12/10: 11 ♀♀ (dissected ♀ RBINSc COP 10.316A-B, preserved material RBINSc COP 10.317; 29/10/2011: 33 ♀♀, 1 juvenile (preserved material RBINSc COP 10.342).
- (2) Pobè, Plateau District, Ouémé drainage basin:
 - Well Pb6: 07/02/11: 4 ♀♀ (preserved material RBINSc COP 10.332); 27/07/2012: 4 ♀♀, 2 ♂♂ (♀ dissected RBINSc COP 10.385A-B, preserved material RBINSc COP 10.336); 26/11/12 : 2 ♀♀ (preserved material RBINSc COP 10.374); 04/03/2013: 3 ♀♀, 3 ♂♂, 2 juveniles (preserved material RBINSc COP 10.345); 20/09/2013: 5 ♀♀, 1 CV copepodite (preserved material RBINSc COP 10.375);
 - Well Pb7: 27/7/12: 15 ♀♀, 4 ♂♂ (dissected ♀ RBINSc COP 10.325A-B, preserved material RBINSc COP 10.326); 16/04/2013: 28 ♀♀, 2 ♂♂, 1 juvenile (preserved material RBINSc COP 10.346);
 - Well Pb 8: July 2013: 2 juveniles (preserved material RBINSc COP 10.343);
 - Well Pb 9: 17/04/2013: 2 ♀♀ (preserved material RBINSc COP 10.341);
 - Well Pb10: 27/07/2012: 2 ♀♀ (preserved material RBINSc COP 10.347); 30/09/2012: 2 ♀♀, 2 ♂♂ (preserved material RBINSc COP 10.384); 25/11/2012: 3 ♀♀ (preserved material RBINSc COP 10.383);
 - Well Pb11: 27/07/2012: 68 ♀♀, 3 ♂♂ (preserved material RBINSc COP 10.344); 30/09/2012: 387 F 5 CV females; 23/02/13: 3 ♀♀, 6 CIV juvenile ♀♀, 4 CV juvenile ♀♀ (dissected adult ♀♀ RBINSc COP 10.322A-C and COP 10.323A-C, preserved material RBINSc COP 10.324);
- (3) Porto-Novo, Ouémé District, Ouémé drainage basin:
 - Well SA: 01/01/10: 8 ♀♀, 1 CIV ♀ juvenile (dissected ♀ RBINSc COP 10.318A-B, preserved material RBINSc COP 10.319); 24/10/2010: 625 adult females and males (preserved material RBINSc COP 10.382);
 - Well AH: 01/11/2009: 12 ♀♀ with body covered with stalked ectoparasites (preserved material RBINSc COP 10.329 and COP 10.330); 30/03/2013: 1 ♀ (preserved material, RBINSc COP 10.380); 31/01/2014: 6 ♀♀ 2 CV juveniles (preserved material, RBINSc COP 10.381);
 - Well AR: 09/12/2010: 1 ♀ (material preserved RBINSc COP 10.331); June 2013: 1 ♀, damaged (preserved RBINSc COP 10.379);
 - Well LB: 20/01/2014: 83 ♀♀ 5 ♂♂ 6 copepodites (damaged, material preserved RBINSc COP 10.373); 09/03/2014: 5 ♀♀ (material preserved RBINSc COP 10.374).

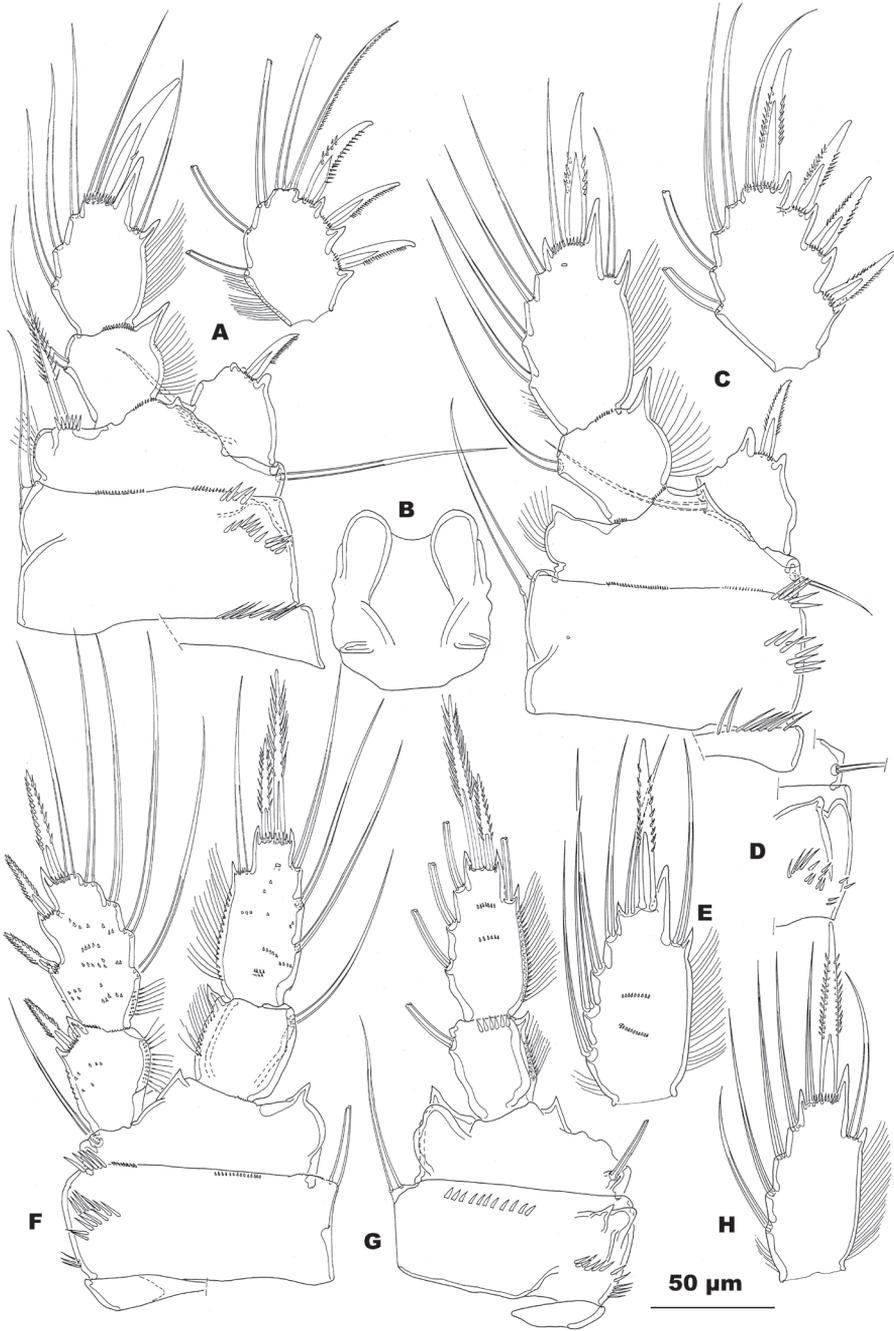


Figure 6. *Alloctyclops spinifer* sp. n. **A** leg 1, frontal view **B** intercoxal sclerite of leg 1, frontal view **C** leg 2, frontal view **D** outer region of coxa and basis of leg 2, caudal view **E** distal segment of leg 3 endopodite, caudal view **F** leg 4, frontal view **G** leg 4 protopodite and endopodite, caudal view **H** distal endopodite segment of leg 3, frontal view. (Female RBINSc COP 10.306: **A, B, C, G**; female RBINSc COP 10.304: **D, F, E**; male RBINSc COP 10.305: **H**).

(4) Kéwi, Kétou District, Ouémé drainage basin:

– village well: 08/02/11: 17 ♀♀, 1 ♂ (preserved material RBINSc COP 10.315).

Description. *Female.* Body (Fig. 7A) typically cyclopid with prosome and urosome almost equally long. Prosome less prominent than in preceding species. Body length: 745 μm (730–820 μm , $n=5$). Laterocaudal expansion of urosomite 1 (pediger 5) accentuated by outwards directed leg 5 (Fig. 8A). Genital double-somite widest in anterior third, tapering caudally. Length/width ratio 1/1. Urosomites 4–5 parallel sided. Cephalothorax, metasomites and first urosomite with narrow and straight posterior fringe. Posterior fringe of genital double somite and urosomites 4–5 narrow and delicately serrate. Surface of somites smooth. Anal somite (Fig. 7D) with continuous row of spinules along posterior margin. Anal operculum crescent, not expanded. Anal sinus with single oblique row of minute spines on both sides.

Caudal rami (Fig. 7D) cylindrical with large mediodorsal triangular depression and 2.7 times as long as wide. Lateral anterior seta short, inserted at caudal end of median third. Distolateral element longer than ramus (ratio: 1/1.2–1.3), furnished with long slender and widely spaced spinules along outer side of stem, and widely spaced setules along medial side. Medial terminal element longer than distolateral one (ratio: 1/0.75) and ramus (ratio: 1/0.65). Breaking plane in both principal terminal setae present; each seta pinnate. Dorsal seta located in caudal third, near medial edge, articulating on short basal part and as long as medial terminal seta. Medial depression with two series of slim spinules (hairlike) arranged perpendicularly on principal axis of the rami. Posterolateral element with spinules at insertion, anterolateral one without.

Antennule 11-segmented with typical armature distribution. First segment with crescent row of slender spinules in proximal half (Fig. 4D). Aesthetasc on segment 8 as long as accompanying seta, linguiform, and reaching almost to distal margin of segment 9. Aesthetasc on segment 10 filiform, shorter than accompanying element and reaching beyond middle of terminal antennular segment. Terminal aesthetasc as long as segments 9–11 combined, fused at base with seta. The latter longer than aesthetasc (Fig. 4C).

Antennal general aspect and armature as in *A. cavicola*. Frontal surface of coxobasis with diffuse pattern of minute spinules in proximal half on and near outer margin (Fig. 5D). Caudal surface with 3 short rows of tiny spinules in proximal half: 1 near outer margin, 1 below a diffuse distal cluster of tiny spinules, and 1 near inner margin (Fig. 5E, F).

Mandible, maxillule and maxilliped as in *A. cavicola*. General aspect of maxilla as in *A. cavicola*. Medial claw of basis rather short and sharp, furnished with 4 large teeth increasing in length medially (Fig. 4K). Accessory element as long as claw, furnished with widely spaced spinules in proximal half, densely serrate in distal half. First endopodite segment with one smooth and one serrate element. Terminal element on second endopodite segment confluent with segment, with long widely spaced spinules in proximal half and densely serrate in distal half.

Legs 1–4 protopodite (Fig. 9A, C, F, G). Praecoxa of legs 1–3 completely devoid of ornaments, of leg 4 with a short row of spinules along distal frontal margin, near outer edge. Both sides of the coxa of legs 1–3 unadorned, except for short rows of min-

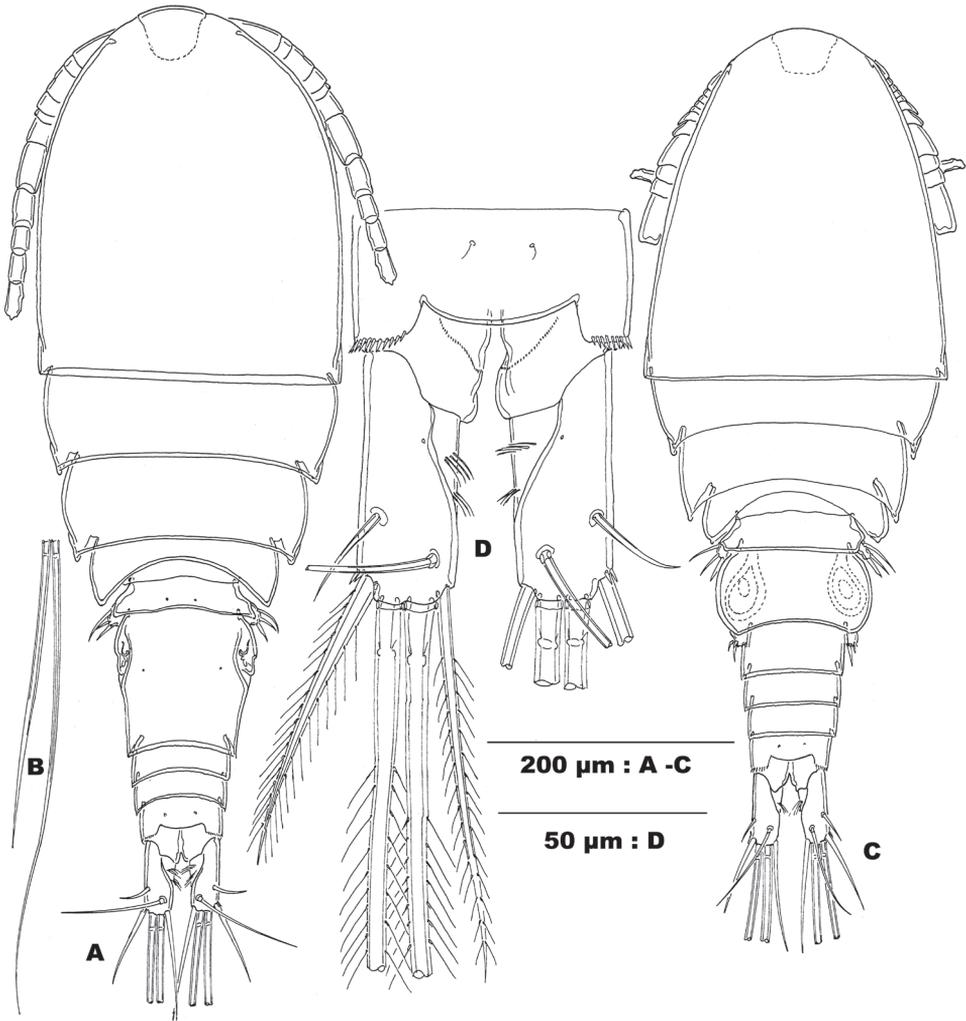


Figure 7. *Alloctyclops pilosus* sp. n. **A** habitus in dorsal view **B** principal caudal setae of left caudal ramus **C** habitus in dorsal view **D** anal somite and caudal rami in dorsal view (Female RBINSc COP 10.310: **A, B, D**; male RBINSc COP 10.312: **C**).

ute spinules along the frontal distal margin. Leg 4 coxa with some spinules on the outer distal frontal edge. Caudal surface of leg 4 coxa with 2 short rows of narrow spinules near the proximal outer caudal edge, and one median row parallel with distal margin. Intercoxal sclerites smooth on both sides, with crescent unadorned laterodistal expansions in legs 1–3 (see ex. Fig. 9B). Leg 4 intercoxal sclerite (Fig. 9H) with concave distal margin, lacking laterodistal expansions. Medial coxal element present in all legs, pinnate and rather short, reaching slightly beyond the distal edge of the basis in legs 1–3, being shorter in leg 4. Medial element on leg 1 basis serrate and long, reaching

to upper quarter of distal endopodite segment. Medial margin of leg 1 basis crescent and hairy. Outer seta of leg 1 basis longer than exopodite. Medial margin of legs 2–3 basis hairy, naked in leg 4. Distal inner edge of basis of legs 2–4 with short triangular expansion. Outer element on basis of leg 2 short, of legs 3–4 long.

Legs 1–4 with 2-segmented rami. Exopodite and endopodite of the legs equally long (Fig. 9A, C, F, G). Armament distribution and general aspect of segments as in preceding species. Segments devoid of ornamentation on frontal and caudal surface. Proximal endopodite segment of leg 4 flat, not expanded caudally. Leg 4 distal endopodite segment 2.2–2.3 times longer than wide with inner terminal spine slightly shorter than segmental length. Outer terminal spine half as long as inner one. Outer subdistal seta reaching to or just beyond tip of inner terminal spine, inner subdistal seta reaching far beyond tip of inner terminal spine. Proximal inner seta shorter than segment.

Leg 5 (Fig. 8A, B) confluent with pediger, represented as a single semi-circular expansion. Ancestral basal segment obsolete, represented by long pinnate seta. Ancestral distal segment represented by two equally long elements: outer one setiform, inner one spiniform.

Leg 6 (Fig. 6B) positioned laterally. Valve surface unadorned and bearing three elements on outer caudal edge: outer one setiform, median and medial one minute and truncate. Genital complex with copulatory pore leading to U-shaped duct. Lateral arms straight. Anterior receptacle expanded, with straight frontal margin. Posterior receptacle bi-lobed, expanded far caudally.

Male. Prosome and urosome equally long with metasome narrower than in the female (Fig. 7C). Urosomite 2 or pediger 6 (Fig. 8C) wide and short (W/L ratio: 2/1). Posterodorsal fringe of prosomites and posterior fringe of urosomites as in the female. Caudal rami with medial hairy ornamentation and armament as in the female. Body length: 668 μm (allotype, paratypes 665–700 μm , n=5).

Antennule as described for *A. cavicola* in Fiers (2012) with 3 long linguiform aesthetascs on segment 1. Antennary armament and spinule pattern, mouthparts and legs 1–4 as in the female.

Leg 5 (Fig. 8C) as in the female. Leg 6 (Fig. 7 C) represented by large semi-ovate unadorned valve. Outer caudal edge expanded, bearing two elements: outer one setiform, medial one spiniform. The former shorter than the latter.

Variability: In most cases the medial triangular depression of the caudal rami is furnished with two parallel sets of fragile hairs as shown in Fig. 7D. In some specimens an additional row is present. Apart from the ornamentation of the caudal rami a single female shows an aberrant armament of the distal endopodite of right leg 2 with only 3 medial setae instead of 4. Body length and slightly different ratios of the caudal rami and armament are noticeable. Female body lengths range from 720 μm (Well A1 population) to 835 μm (Kéwi population); males from 620 (Well A1 population) to 800 μm (Kéwi population). Length/width ratio of the caudal rami varies between 2.4 to 3.0 with the laterodistal spine 1.2–1.4 and the terminal medial seta 1.6–1.9 times longer than the rami.

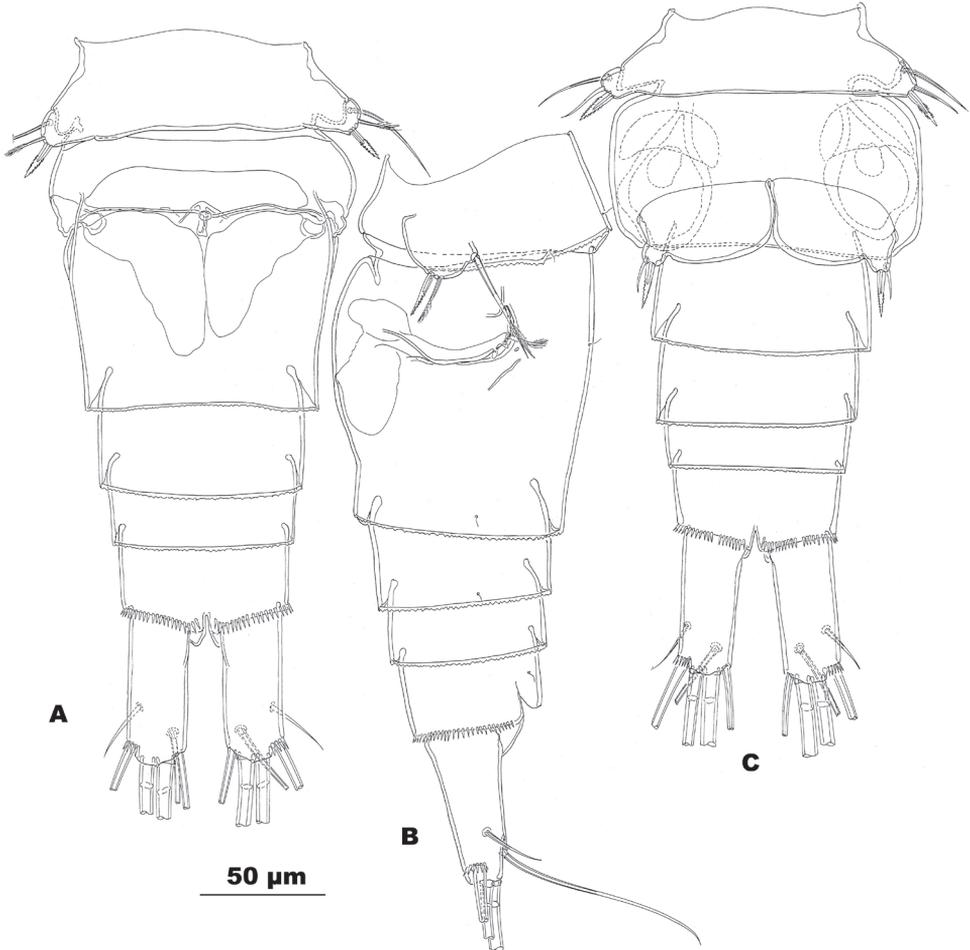


Figure 8. *Allocyclops pilosus* sp. n. **A** urosome in ventral view **B** urosome in lateral view **C** urosome in ventral view (Female RBINSc COP 10.310 **A, B**; male RBINSc COP 10.312: **C**).

***Allocyclops nudus* sp. n.**

<http://zoobank.org/9231A518-6786-4A71-ACFE-5AD9DE002D62>

Figs 4E, F, J, 5G, H, 10, 11, 12

Syn. *Allocyclops* n sp. [*partim*] in Lagnika et al. 2014b

Type locality. Ladjifarani or “Nouveau quartier”, Parakou District, Ouémé drainage basin: Well B6 (details in Table 2).

Type material. Holotype female, dissected and mounted on 2 slides, RBINSc COP 10.334A-B, allotype male, dissected and mounted on 2 slides, RBINSc COP 10.335A-B; paratypes: dissected female RBINSc 10.367A-B, preserved material: 7 females, 3 males, 5 juveniles (RBINSc COP 10.333) all collected 29/10/2011; addi-

tional paratypic material (topotypes): 3 females collected 16/01/11 (preserved RBINSc COP 10.352).

Etymology. From the Latin adjective “*nudus*” meaning naked and refers to the absence of hairy or spinular ornamentation on the pedigers surface and caudal rami, contrasting the species from *A. spinifer* sp. n. and *A. pilosa* sp. n.

Additional material.

(1) Parakou District, Ouémé drainage basin.

- Well A1: 27/08/2011: 1 ♀, 8 ♂♂ (preserved material RBINSc COP 10.337); 11/01/2011: 4 ♀♀, 1 ♂, 40 juveniles, decomposed condition (preserved material RBINSc COP 10.348); 15/02/2011: 2 ♀♀, 3 ♂♂, 20 juveniles (preserved material RBINSc COP 10.351); 10/07/2012: 48 ♀♀, 12 ♂♂ (preserved material RBINSc COP 10.338);
- Well A5: 16/11/2010: 2♂♂, 4 juveniles (preserved material RBINSc COP 10.350);
- Well C3: 30/10/2011: 1 ♂ (dissected male RBINSc COP 10.368); 14/02/2011: 27 ♀♀, 1 ♂ (preserved material RBINSc COP 10.349).

Description. *Female.* Body (Fig. 10A) typically cyclopid with prosome longer than urosome (ratio: 1/0.8). Principal body flexure well marked, rather wide. Genital double-somite widest in anterior third, tapering caudally, and as long as widest part. Anal somite with crescent operculum, not expanded. Anal sinus unadorned. Length 900 µm (holotype, paratypes 890–962 µm, n=5). Posterodorsal margins of cephalothorax and pedigers 2–3 straight, of pediger 4 slightly irregular. Urosomite 1 with straight posterodorsal fringe. Posterior margin of genital double-somite and urosomites 4–5 wide, coarsely serrate. Posterior margin of anal somite with uninterrupted row of spinules along ventral and dorsal margins.

Caudal rami cylindrical with triangular depression along anterior half of medial margin, less than 3 times longer than wide (L/W-ratio 1/2.7–2.8). Anterolateral seta, pinnate, short, without spinules near articulation. Posterolateral element shorter than ramus in holotype (ratio: 0.9/1) ranging from 0.8/1–1.0/1 in paratypes), with long and slender spinules along outer side of the stem, pinnate along the inner side. Spinules present at insertion with ramus. Medial element longer than ramus (ratio: 1.15–1.25/1) and pinnate. Principal terminal setae with breaking plane and pinnate. Dorsal seta 1.3–1.4 times longer than ramus, and articulating on small basal part. Integument of rami unadorned.

Antennule 11-segmented with typical armament, not reaching to posterolateral edge of cephalothorax. Segment 1 with set of slender spinules and set of short ones (Fig. 4 F). Aesthetasc on segment 8 linguiform, reaching beyond middle of segment 9. Aesthetasc on segment 10 filiform, shorter than accompanying seta and reaching halfway terminal segment. Aesthetasc on segment 11 tubular, as long as segments 9–11 combined, and shorter than accompanying seta (Fig. 4E).

Antenna with general appearance and armature as in *A. chappuisi* (see Fiers 2012). Frontal surface of coxobasis with a cluster of 7–9 small spinules near middle of outer margin and a row of slender spinules in proximal half near abexopodal margin (Fig. 5G). Caudal surface of coxobasis with 3 sets of narrow spinules in proximal half: 2 near outer margin, 1 near abexopodal margin (Fig. 5H).

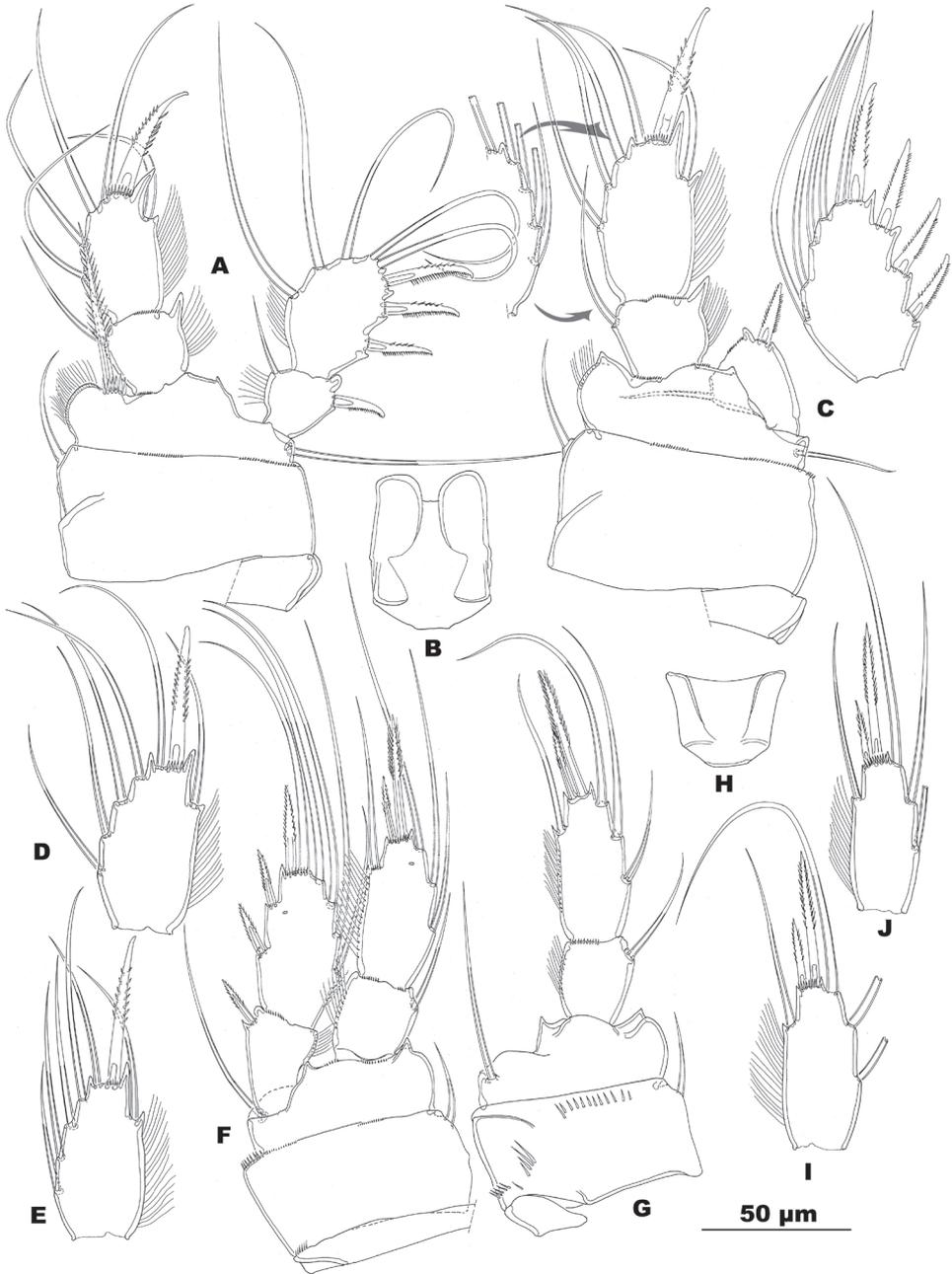


Figure 9. *Allocyclops pilosus* sp. n. **A** leg 1, frontal view **B** leg 1 intercoxal sclerite **C** leg 2, frontal view (aberrant number of medial setae on endopodite, normal seta complement shown at the left) **D** distal endopodite segment of leg 3, frontal view **E** idem **F** leg 4, frontal view **G** leg 4, protopodite and endopodite, caudal view **H** leg 4 intercoxal sclerite, frontal view **I–J** distal endopodite segment of leg 4 of other specimens (Females RBINSc COP 10.313: **F, G**; COP 10.310: **A–D, G–H**, COP 10.318: **I**; COP 10.316: **J**; male RBINSc COP 10.312: **E**).

Mouthparts as in preceding species. Maxillary basis (Fig. 4J) prominent and blunt, with set of 4–5 large spinules in middle, increasing in length medially. Accessorial seta robust, with large spinules on both sides of the stem in proximal half, finely serrate along one side in distal half. Armature of proximal endopodite segment and terminal armature element on distal endopodite segment robust, with large spinules in proximal half and finely serrate in distal half on one side of the stem. Terminal seta on distal endopodite segment confluent with segment. Additional elements on distal segment sparsely serrate.

Leg 1–4 protopodite (Fig. 12A–D). Frontal margin of praecoxa with short row of slender spinules near outer corner. Frontal surface of legs 1–4 coxa unadorned except for some minute spinules along distal margin. Caudal surface of leg 1 unadorned, of legs 2 and 3 with some slender spinules near outer proximal corner. Caudal surface of leg 4 coxa with a transverse row of spinules parallel to distal margin and some slender spinules near outer proximal corner. Intercoxal sclerite of legs 1–4 as in preceding species. Medial coxal seta present, pinnate and reaching beyond basis, attending distal margin of proximal endopodite segment in leg 1, and middle of proximal endopodite segment in legs 2 and 3. Medial element somewhat shorter in leg 4. Medial margin of basis of leg 1 crescent, hairy, and with pinnate element reaching beyond proximal endopodite segment. Spinules present at its insertion. Medial margin of basis of legs 2–4 crescent, produced distally in short triangular expansion. Hairy in legs 2–3, naked in leg 4. Outer seta on leg 1 basis as long as exopodite, and as long as first exopodite segment in legs 2–4.

Leg 1–4 rami 2-segmented with general appearance and armament distribution as in preceding species. Frontal and caudal surface of ramal segments unadorned. Proximal segment of leg 4 endopodite inflated caudally (Fig. 12D). Distal endopodite segment of leg 4 2.10–2.15 times longer than wide. Inner terminal spine as long as segment, and 1.8–2.1 times longer than outer spine. Medial setae on second endopodite segment equally long, not reaching beyond inner terminal spine.

Leg 5 (Fig. 11E) with ancestral segments confluent with pediger, represented as a semi-ovate expansion with a discreet truncation distally. Ancestral basal segment represented by a long setiform element. Ancestral distal segment represented by 2 elements: outer one setiform, half as long as medial spiniform element, both inserted on the distal discreet truncation.

Leg 6 (Fig. 11B) typically represented by 3 elements: outer one setiform, middle and medial ones dwarfed, hyaline and blunt. Valves unadorned. Genital complex with frontal and caudal parts of receptacles expanded. The former with undulate frontal margin, the latter with single medioventrally expansion. Copulatory pore leading to U-shaped duct (Fig. 11D). Lateral arms straight.

Male: Body narrower than female body (Fig. 10C). Prosome 1.5 times longer than urosome. Principal body flexure wide. Second urosomite (pediger 6) 2 times wider than long. Body length 795 μm (allotype, ranging from 744 to 817 in paratypes). Posterodorsal margins of prosomites and first urosomite narrow and straight. Posterior hyaline fringe margins of urosomite 2 narrow and serrate, of urosomites 3–5 wide and

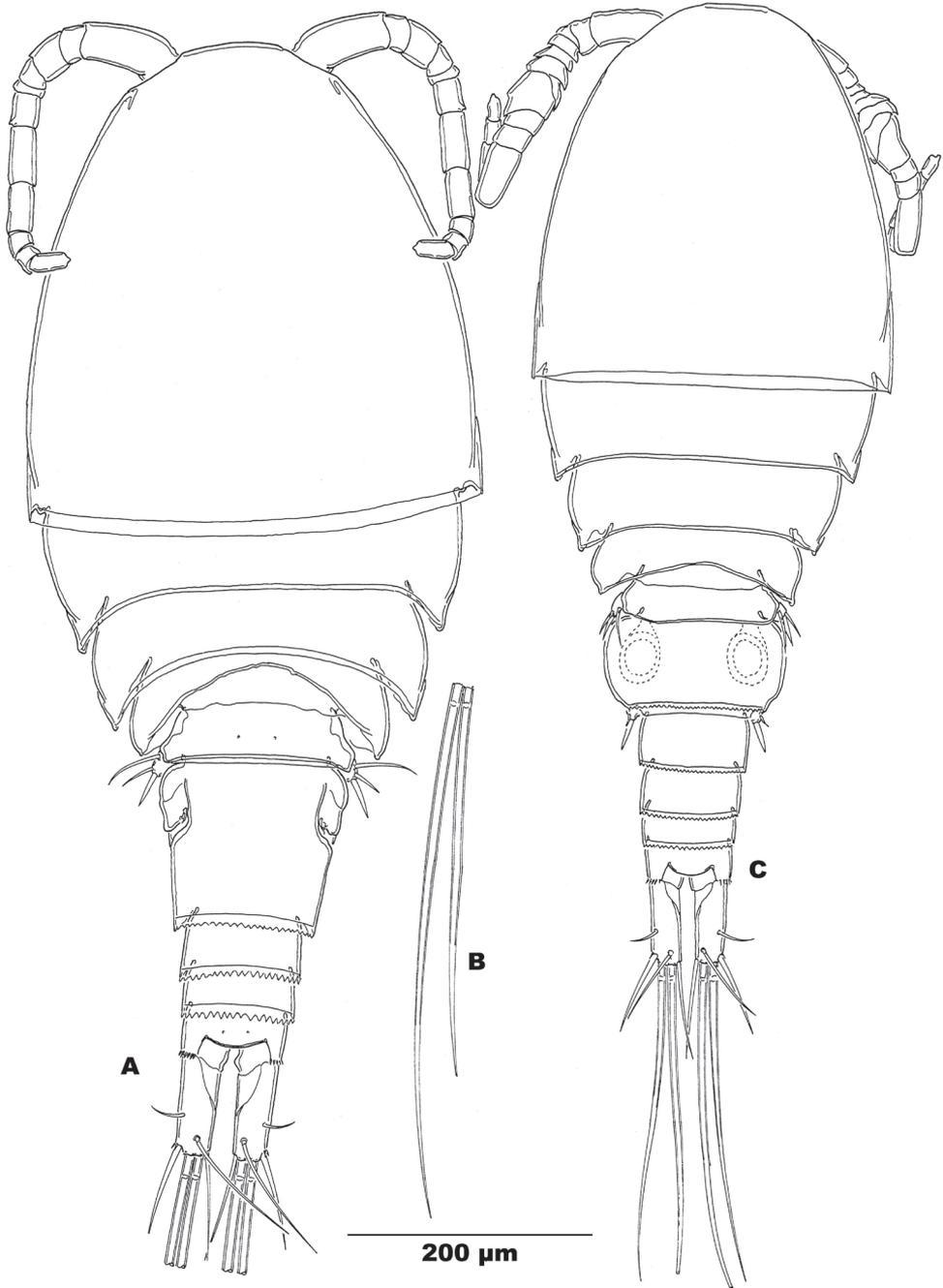


Figure 10. *Alloccyclops nudus* sp. n. **A** habitus in dorsal view **B** principal caudal setae of right caudal ramus **C** habitus in dorsal view (Female RBINSc COP 10.334: **A**, **B**; male RBINSc COP 10.335: **C**).

serrate. Anal somite with crescent, not expanded, anal operculum. Anal sinus smooth. Posterior margin of anal somite with uninterrupted row of spinules.

Caudal rami cylindrical with short triangular medial depression in anterior half. L/W-ratio: 2.7/1 (allotype, ranging from 2.7–3.0/1 in other male paratypes). Antero-lateral seta short, pinnate, without spinules at insertion. Distolateral element shorter than ramus (ratio: 0.8–0.9/1) serrate along outer margin, pinnate along inner one. Spinules at insertion present. Terminal medial element longer and dorsal one longer than ramus: 1.15–1.25/1 and 1.25–1.35, respectively. Dorsal seta articulating on basal part. Principal terminal setae with breaking plane and pinnate.

Antennule as described for *A. cavicola* in Fiers 2012. Mouthparts and legs 1–4 as in the female, except for leg 4 endopodite lacking expansion of caudal surface of the proximal segment. Leg 5 (Fig. 11C) with region representing ancestral distal segment more pronounced than in the female. Armature elements as in the female.

Leg 6 (Fig. 11C) represented as large, unadorned valve with caudally produced outer distal edge bearing 2 elements: outer one very short, setiform, medial long, spini-form and serrate.

Variability. Except for slight variation (less than 15 µm) in the body length and the L/W ratio of the caudal rami and its armament, no structural differences were observed in the type series nor in the other populations.

***Allocyclops saktii* sp. n.**

<http://zoobank.org/316EB4BF-2BA4-406E-B289-81CE29B3C34D>

Figs 4G, H, L, 5I, J, 13, 14, 15

Type locality. at Addrodji, Lokossa District, Mono Couffo drainage basin: Well P2 (details in Table 1).

Type material. Female holotype, dissected and mounted on 3 slides, RBINSc COP 10.354; allotype male, dissected and mounted on 2 slides, RBINSc COP 10.356; female paratypes dissected: RBINSc COP 10.355A-C, COP 10.357 A, COP 10.358 A-C; 14 females and 5 males preserved (RBINSc COP 10.359), all collected 1-12-2012. Additional paratypic material (topotypic), collected 19/06/2012: 185 females, 15 males (preserved material RBINSc COP 10.360).

Etymology. Named after Nestor G. Sakiti who initiated, in collaboration with Claude Boutin, subterranean biology research in Bénin, and supported the junior author initializing his investigations.

Additional material.

(1) Lokossa, Mono District, Mono-Couffo drainage basin:

- Well P6: 17-06-2012: 1 ♀ (preserved material RBINSc COP 10.364); 06/09/2012: 18 ♀♀ 1 ♂ (preserved material RBINSc COP 10.378); Well PM (about 20 m from well P6, cemented, closed and abandoned, and considered here as identical with well P6): 07/01/2012: 65 ♀♀, 4 ♂♂ (dissected ♀ RBINSc COP 10.370A-C; preserved material RBINSc COP 10.365).

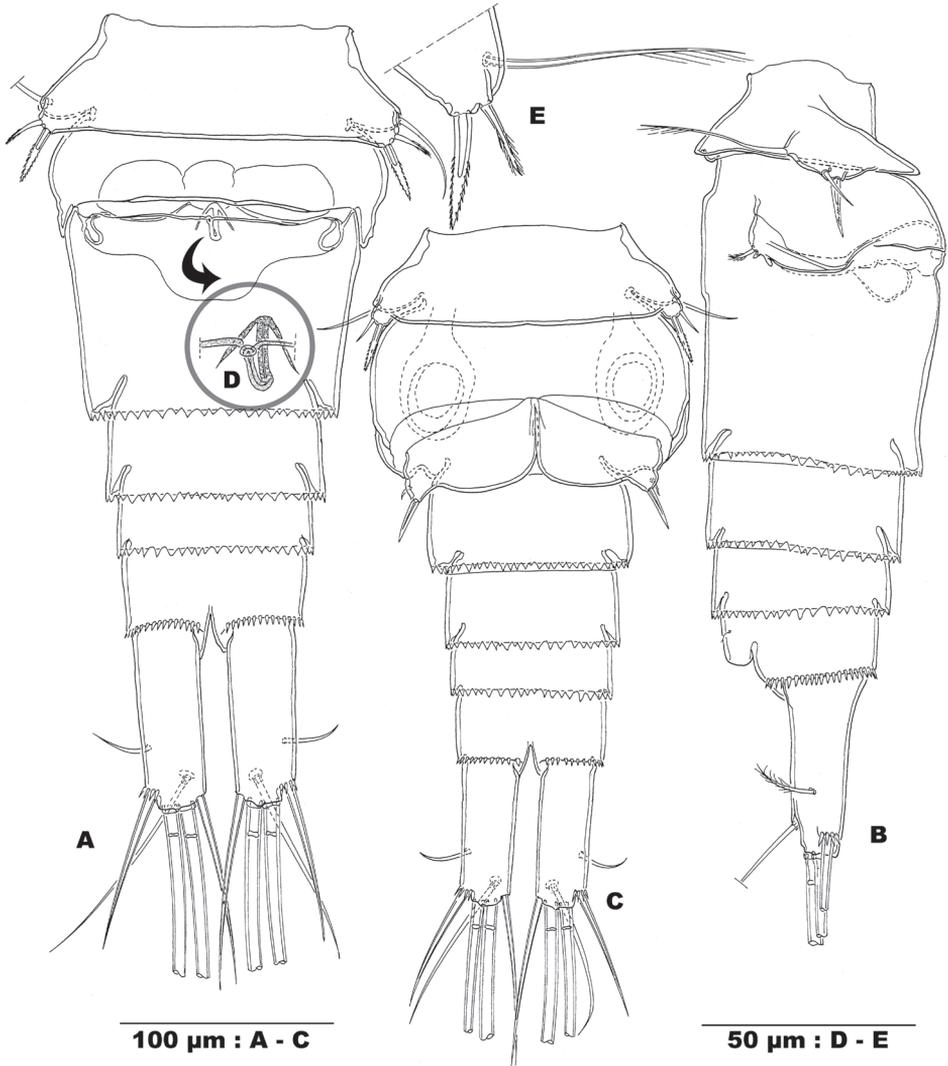


Figure 11. *Alloccyclops nudus* sp. n. **A** urosome in ventral view **B** urosome, lateral view **C** urosome in ventral view **D** copulatory pore and duct **E** leg 5 and outer edge of pediger 5 (Female RBINSc COP 10.334: **A, B, D, E**; male RBINSc COP 10.335: **C**).

- Well P7: 13-06-2012: 74 ♀♀, 4 ♂♂, 2 juveniles (dissected ♀ RBINSc COP 10.369A-C; preserved material RBINSc 10.363);
 - Well P9: 11-06-2012: 116 ♀♀, 11 ♂♂, 9 juveniles (preserved material RBINSc COP 10.361); 30-11-2012: 9 ♀♀, 2 ♂♂ (preserved material RBINSc COP 10.362).
- (2) Abomey-Calavi District, Ouémé drainage basin:
- Well near Campus: 27-02-2013: 24 ♀♀, 3 ♂♂, 1 juvenile (preserved material RBINSc COP 10.366).

Description. *Female:* Body (Fig. 13A) typically cyclopid with prominent prosome (1.8–1.9 times urosome length). Genital double-somite widest in anterior third, and as long as widest part. Posterodorsal fringe of prosomites and urosomite 1 narrow and straight. Posterior fringe of genital double-somite and urosomites 3–4 narrow and discretely serrate (Fig. 14A, B). Anal somite with shallow crescent operculum (Fig. 13C). Anal sinus apparently smooth. Posterior margin of anal somite with uninterrupted row of spinules: rather short dorsally, laterally and along outer half of ventral margin, twice as long along medial half of ventral margin (Fig. 14A). Body length: 833 μm (holotype, paratypes ranging from 807–876 μm , n=5).

Caudal rami (Fig. 13C) cylindrical, between 2.5 and 2.6 times longer than wide, with triangular medial depression reaching halfway the margin. Integument of caudal rami smooth. Anterolateral seta short and pinnate, without spinules at insertion. Distolateral element equally or slightly longer than ramus (ratio: 1.0–1.1/1), furnished with widely spaced setules on both sides of the stem. Spinules at insertion of distolateral element present. Terminal medial element longer than ramus (ratio: 1.3–1.5/1) and pinnate. Principal terminal setae with breaking plane and pinnate. Dorsal seta, articulating on small basal part, and as long as medial element.

Antennule 11-segmented, not reaching to posterior edge of cephalothorax. Segment 1 with crescent row of spinules in proximal half (Fig. 4H). Aesthetasc on segment 8 linguiform, as long as accompanying seta, and reaching middle of segment 9. Aesthetasc on segment 10 filiform, as long as accompanying seta, and reaching nearly to distal end of terminal segment. Aesthetasc on segment 11 tubular, as long as segments 9–11 combined, and shorter than accompanying seta (Fig. 4G).

Antennary general morphology and armature as in *A. chappuisi*. Frontal surface of coxobasis with a cluster of minute spinules (6–7 spinules) in middle of outer margin and a short transverse set of slender ones in the proximal half bear the abexopodal margin (Fig. 5J). Caudal surface nearly completely naked only furnished with a few slender spinules in the proximal half near the abexopodal margin. (Fig. 5I).

Mandible, maxillule, maxilliped and general aspect of maxilla as in *A. cavicola* and the preceding species. Claw of maxillary basis blunt, armed with 8–9 large spinules, increasing in width and length medially. Claw shorter than accessory element (Fig. 4L). The latter furnished with groups of long spinules on both sides in proximal half and along one side in distal half, besides the finely serrate ornamentation of the distal part of the stem. Both elements on proximal endopodite segment and terminal element on distal segment furnished partially with long spinules along one side of the stem, finely serrate at the opposite side. Additional setae on distal endopodite segment slender, unequal in length.

Leg 1–4 protopodite without ornamentation on praecoxa (Fig. 15A, C, E, F). Frontal surface of coxa unadorned except for the usual minute spinules along distal margin. Caudal coxal surface smooth in leg 1, with some slender and short spinules near outer proximal corner in legs 2 and 3 (Fig. 15D). Caudal surface of leg 4 coxa with 2 small clusters of spinules near outer proximal distal corner and a median row of narrow spinules parallel with distal margin (Fig. 15F). Medial coxal seta present in

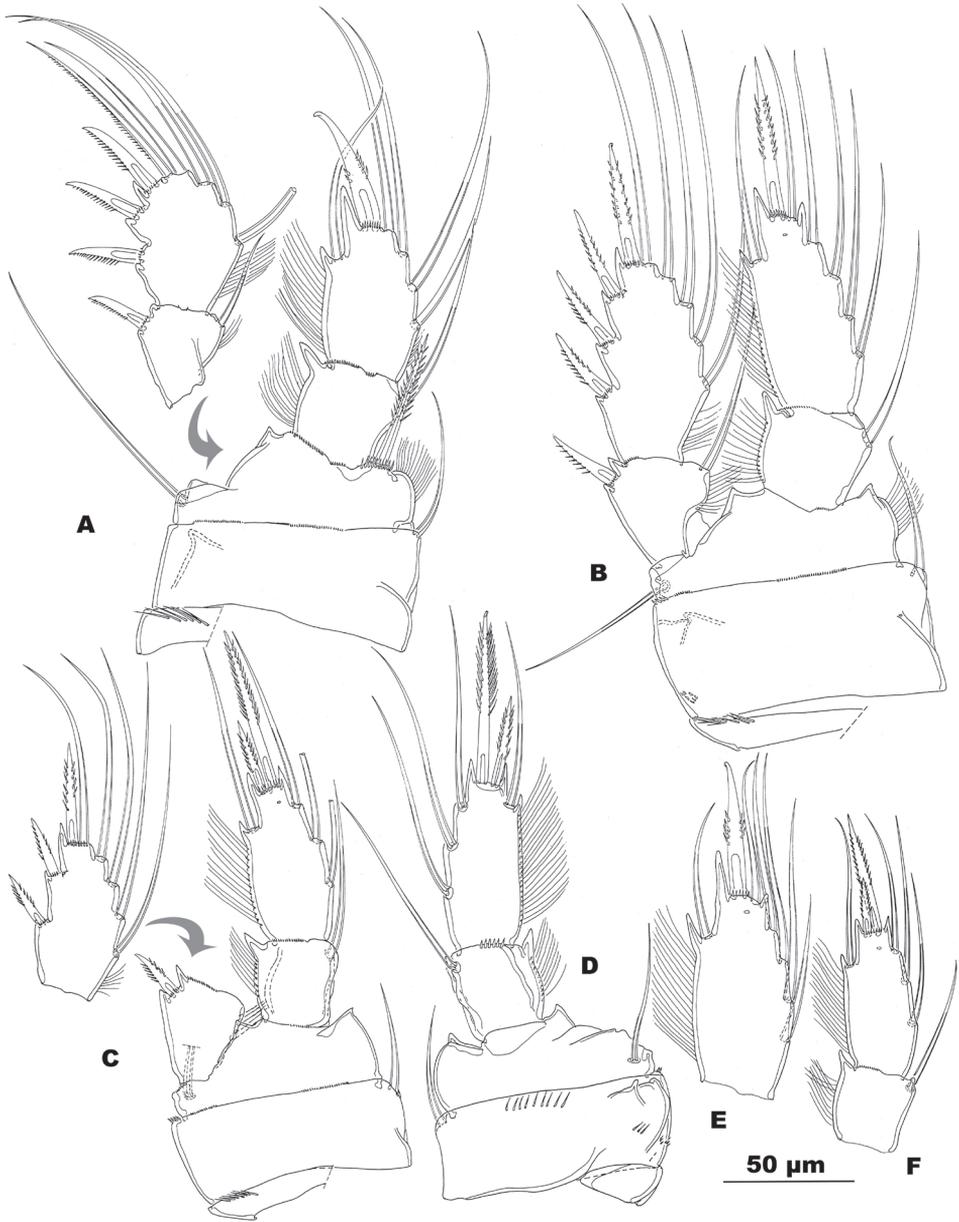


Figure 12. *Alloccyclops nudus* sp. n. **A** leg 1, frontal view **B** leg 2, frontal view **C** leg 4, frontal view **D** leg 4 protopodite and endopodite, caudal view **E** leg 3 distal endopodite segment, frontal view **F** leg 4 endopodite, frontal view (Female RBINSc COP 10.367: **A, C, E**; Female RBINSc COP 10.334: **B, D**; male RBINSc COP 10.335: **E**).

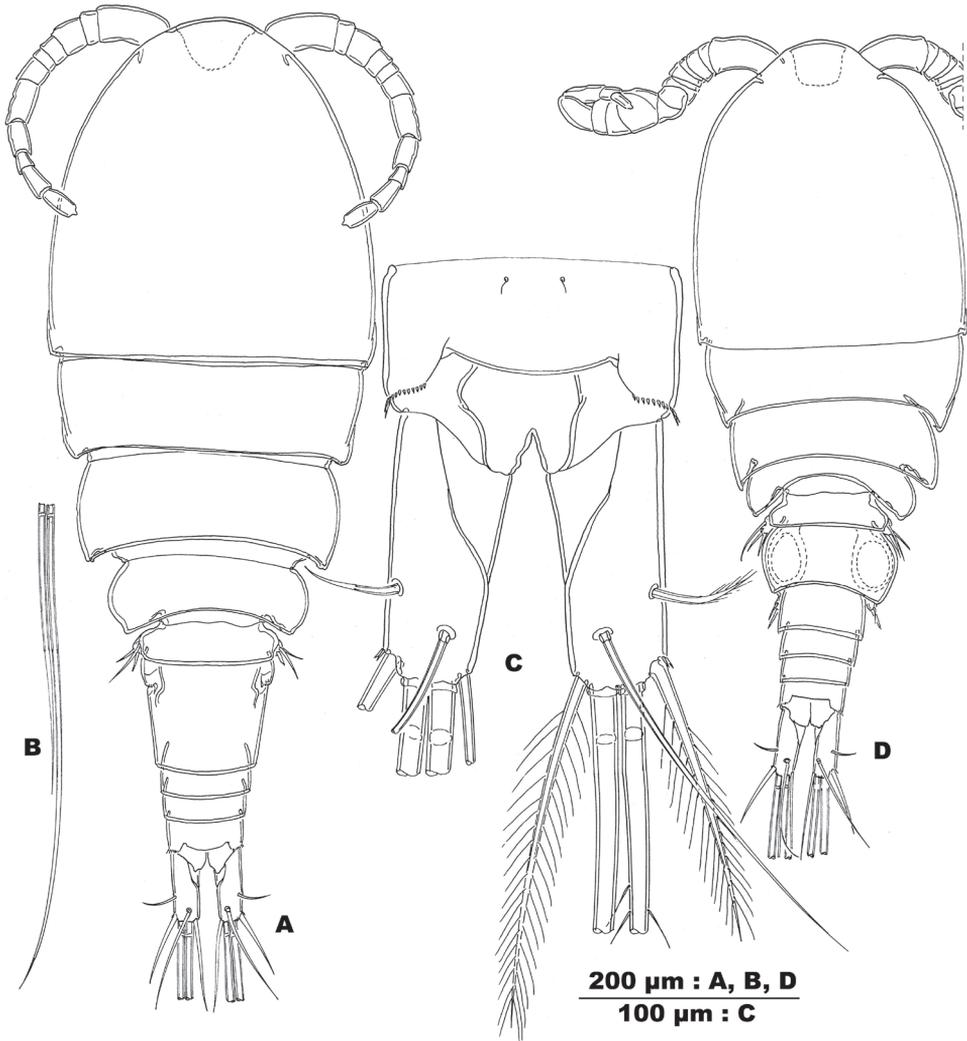


Figure 13. *Allocyclops sakitii* sp. n. **A** habitus in dorsal view **B** principal caudal setae of left caudal ramus **C** anal somite and caudal rami, in dorsal view **D** habitus in dorsal view (Female RBINSc 10.357: **A, B**; female RBINSc COP 10.354: **C**; male RBINSc COP 10.356: **D**).

all legs, pinnate and reaching to middle of proximal endopodite segment in legs 1–3, but hardly beyond basis in leg 4. Intercoxal sclerites as in preceding species. Basis in leg 1 with long medial spine, reaching to middle of distal endopodite segment and with spinules at insertion. Medial margin of legs 1–3 crescent and hairy, naked in leg 4. Medial distal edge of basis in leg 1 rounded, slightly triangularly produced in legs 2–4. Outer seta on basis of legs 1 and 4 as long as exopodite, of legs 2 and 3 half as long.

Leg 1–4 rami 2-segmented with general appearance and armament distribution as in preceding species (Fig. 15A–C, E–F). Proximal segment of leg 4 endopodite slightly

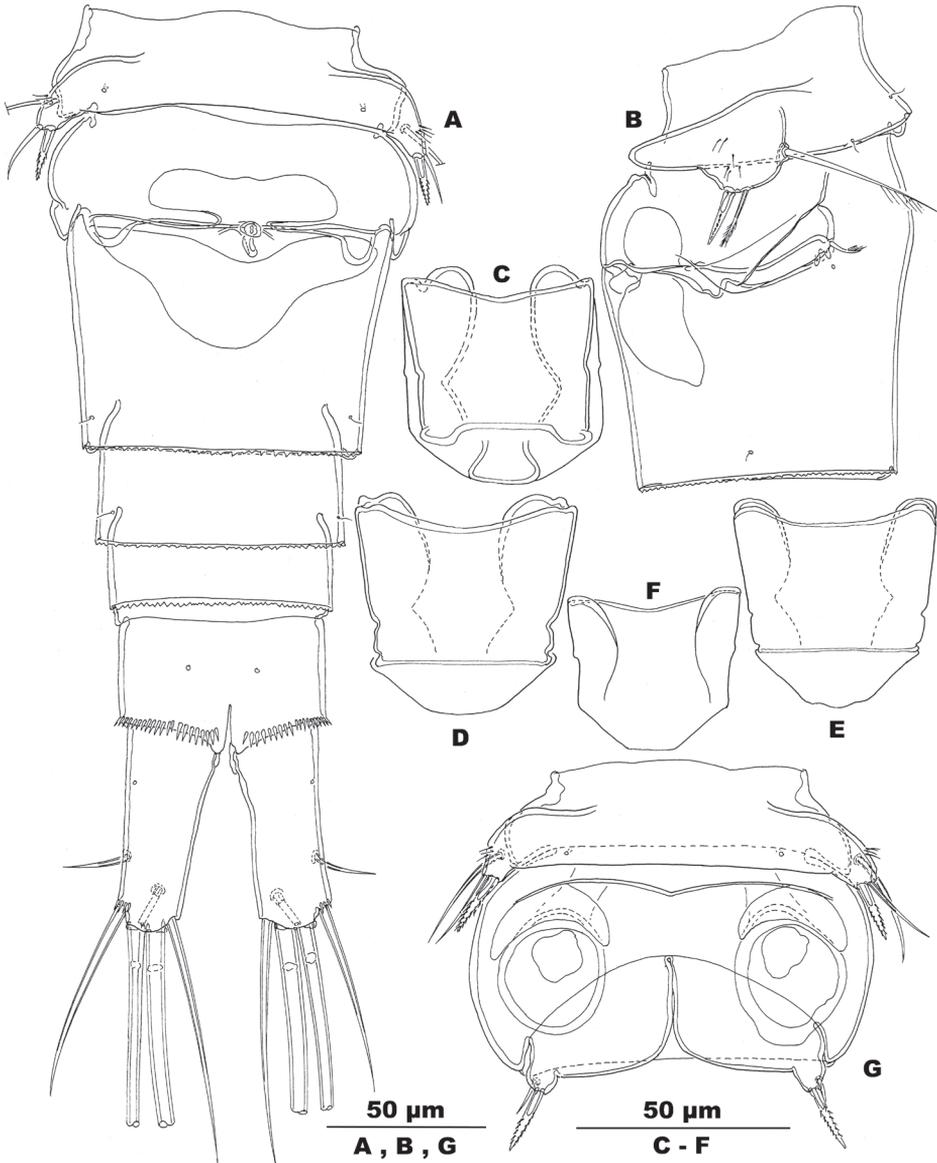


Figure 14. *Allocyclops sakitii* sp. n. **A** urosome, ventral view **B** pediger 5 and genital double-somite, in lateral view **C** leg 1 intercoxal sclerite, frontal view **D** leg 2 intercoxal sclerite, frontal view **E** leg 3 intercoxal sclerite, frontal view **F** leg 4 intercoxal sclerite, frontal view **G** pediger 5 and urosomite 2 in ventral view (Female RBINSc COP 10.355: **A, B**; COP 10.358: **C-F**; male RBINSc COP 10.356: **G**).

expanded caudally. Distal endopodite segment of leg 4 2.2–2.4 times longer than wide. Inner terminal spine shorter than segment (ratio: 0.9/1), outer spine half as long as inner one. Medial setae on distal endopodite segment of leg 4 reaching distinctly beyond longest terminal spine.

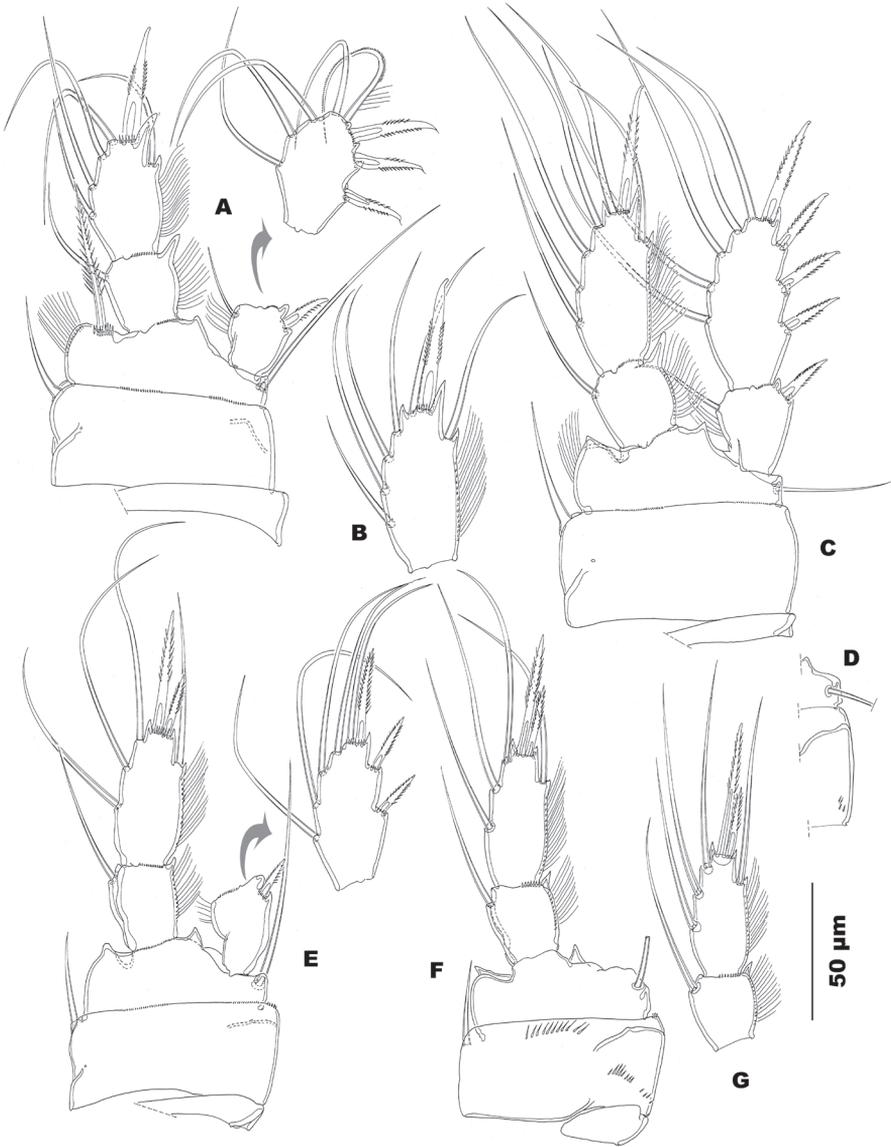


Figure 15. *Allocyclops sakitti* sp. n. **A** leg 1, frontal view **B** distal endopodite segment of leg 2, frontal view **C** leg 3, frontal view **D** Outer region of coxa and basis of leg 3, caudal view **E** leg 4, frontal view **F** leg 4 protopodite and endopodite, caudal view **G** leg 4 endopodite, caudal view (Female RBINSc COP 10.355: **A–C, F**; COP 10.358: **D, E**; male RBINSc COP 10.356: **G**).

Leg 5 (Fig. 14A, B) confluent with pediger, represented as a semi-circular expansion bearing 3 elements. Ancestral basal segment represented by long setiform element, ancestral distal segment by two equally long elements: outer one setiform, inner one spiniform. Surface of leg 5 expansion with some short and hairy-like spinules.

Leg 6 (Fig. 14B) located laterally on genital double-somite, typically with 3 elements: outer one setiform, middle and medial one dwarfed, hyaline and blunt. Valve unadorned. Genital complex with small ovate anterior receptacle. Caudally expanded receptacle with single medioventral lobe. Copulatory pore leading to U-shaped duct. Lateral arms straight.

Male: General appearance of body as in the female with prosome 1.5 times longer than urosome. The latter with pediger 6 half as long as wide (Fig. 14G). Posterodorsal fringes of prosomite and first urosomite, and posterior fringes of urosomites as in the female. Body length: 676 μm (allotype and other male paratypes ranging between 650 and 695 μm ; n=4).

Caudal rami 2.5–2.7 times longer than wide, distolateral element 1–1.15 times longer than caudal rami, and terminal medial element 1.30–1.45 times longer than ramus.

Antennule as described for *A. cavicola* in Fiers (2012). Mouthparts and legs as in the female but proximal endopodite segment of leg 4 without expansion.

Leg 5 (Fig. 14G) with general appearance and integumentary ornamentation as in the female, but with outer setiform element on distal segment vestige longer than inner spiniform element.

Leg 6 vestige (Fig. 14G) large, with protruded outer distal edge, bearing 2 elements: outer one setiform, half as long as inner one. The latter spiniform. Surface of valve unadorned.

Variability: Apart from slight variation in body length and L/W-ratio of the caudal rami and the setae on the caudal rami, no structural variability was observed.

Discussion

Differential diagnoses

The cyclopoid genus *Allocyclops* originally erected by Kiefer (1932) and recently redefined (Fiers 2012) corals four species with three African representatives: *A. chappuisi* Kiefer, 1932, *A. cavicola* Chappuis, 1951 and *A. beadleii* (Lindberg, 1956) and one Central-American (*A. botosaneanui* Pleša, 1981). The latter which deviates in many aspects from its three congeners has been tentatively retained in the genus but will certainly be removed when its affinities to the South and North American cyclopinines becomes clarified (Fiers 2012). The three known African representatives are described from sub-Saharan localities located in the tropical savannah region of continental Africa (Peel et al. 2007): *A. chappuisi* Kiefer, 1932 from Ivory Coast (see Chappuis 1934), *A. cavicola* Chappuis, 1951 from Bas-Congo in the Democratic Republic of Congo (see Leleup 1956), and *A. beadleii* (Lindberg, 1956) from Uganda.

An unidentified cyclopinine attributed to *Allocyclops* was reported from Morocco (Tuekam Kayo et al. 2012). The specimens have been re-examined by the senior author (35 ♀♀, 1 ♂: catalogued: RBINSc COP 9403, COP 9406, COP 10.132). They can, however, not be assigned to the genus *Allocyclops* but seems to be affiliated with *Metacyclops* Kiefer, 1927.

Among the 4 species described here, *A. nudus* sp. n. resembles most closely *A. chappuisi*, type of the genus, and can be easily confounded with the latter. *A. nudus* sp. n. is however more robust and distinctly larger than *A. chappuisi*. Females of the former measure 895–962 μm and males 744–817 μm whereas females and males of *A. chappuisi* measure 720–740 μm and 650 μm , respectively (Kiefer 1932; 1934). The laterodistal spine on the endopodite of leg 4 is less than half the length of the mediolateral element while in *A. chappuisi* it reaches slightly beyond the middle of the inner spine. The most obvious difference between both species are the proportional lengths of the middle seta and medial spine of leg 5 (in both sexes) and leg 6 of their respective males. In *A. nudus* sp. n. the medial spiniform element is distinctly longer than the middle seta in leg 5 and leg 6 whereas in *A. chappuisi* the female leg 5 medial spine is shorter than the middle one, and equally long in the male leg 5 and leg 6.

As for *A. nudus* sp. n., *A. sakitii* sp. n. resembles *A. chappuisi* in most aspects. *A. sakitii* sp. n. differs from the latter by the larger body dimensions (♀ : 800–875 μm , ♂ : 650–695 μm versus ♀ : 720–740 μm , ♂ : 650 μm), the longer caudal rami (L/W: ♀ 2.50–2.65 versus 2.33) and the longer distolateral element of the caudal rami (> than ramal length versus < than ramus). *A. sakitii* sp. n. might also be distinguishable from *A. chappuisi* by the presence of the hairy ornamentation on the lateral margin of pediger 5. However, presence or absence of ornamentation on pediger 5 in *A. chappuisi* could not be explicitly confirmed because of the condition of the material (see Fiers 2012).

A. pilosus sp. n. and *A. spinifer* sp. n. are readily distinguishable from all the other *Allocyclops* species by the presence of ornamentation on the caudal rami: pilose in *A. pilosus* sp. n., serrate in *A. spinifer* sp. n. The pilose structures on the rami of *A. pilosus* sp. n. remind to some extent the ornamentation on the caudal rami of *A. cavicola* but is quite different in the former because of the linear arrangement of the setules and their location restricted to the medial margin of the rami versus the dorsal, lateral and medial location, and the more abundant presence of the structures on the rami of *A. cavicola*.

The morphology of four new species described herein concurs completely with the amended diagnosis of *Allocyclops* as proposed by Fiers (2012). On the basis of their morphology (*i.e.* body shape, leg armament, buccal appendages) Fiers (2012) surmised a close relationship between *Allocyclops* and certain African *Metacyclops* species. The new additions neither dismiss nor support this assumption. The lack of detailed information on the morphology of the African *Metacyclops* species stamps such coherent analyses.

Key to the African species of the genus *Allocyclops*, applicable for both genders

- 1 Leg 4 distal exopodite segment with 5 medial setae; Caudal rami with medial seta twice as long as ramus, at most..... **2**
- Leg 4 distal exopodite segment with 4 medial setae; Caudal rami with medial seta 2.4–2.5 times longer than ramus..... ***A. beadlei***
- 2 Caudal rami 3 times longer than wide at the most (between 2.3–3.0/1); Medial seta on caudal rami 1 to 1.5 times as long as ramus, at the most..... **3**

- Caudal rami more than 3 times longer than wide (generally 3.15/1); Medial seta on caudal rami 2 times as long as ramus..... ***A. cavicola***
- 3 Pediger 5 without ornamentation on lateral and ventrolateral margins.....**4**
- Pediger 5 furnished with either spinules or slender setules on lateral and ventrolateral margins..... **6**
- 4 Medial margin of caudal rami with 2 or 3 transverse rows of thin hairs (visible only when rami are observed dorsally); Leg 4 terminal endopodite segment 2.2–2.3 times longer than wide; Distolateral seta longer than caudal rami (1/1.2–1.3)***A. pilosus* sp. n.**
- Medial margin of caudal rami without ornamentation; Leg 4 terminal endopodite segment 2.1 times longer than wide at the most; Distolateral seta as long as caudal rami at the most (1/0.8–1.0) **5**
- 5 Caudal rami 2.7–2.8 times longer than wide; Dorsal seta on caudal rami 1.5 times longer than ramus; Medial spine of leg 5 1.5 times longer than middle setiform element***A. nudus* sp. n.**
- Caudal rami 2.5 times longer than wide at the most; Dorsal seta on caudal rami less than 1.5 times longer than ramus; Medial spine of leg 5 shorter than middle setiform element***A. chappuisi***
- 6 Lateral and ventrolateral margin of pediger 5 with cluster of spinules; Surface of caudal rami and leg 4 ramal segments with diffuse pattern of minute spinules ***A. spinifer* sp. n.**
- Lateral and ventrolateral margin of pediger 5 with cluster of fine hair-like setules; surface of caudal rami and leg 4 ramal segments unadorned.....
..... ***A. sakitii* sp. n.**

Occurrence

Of the 52 regularly visited wells, 40 (77%) were populated with a copepod fauna: 39 exclusively with Cyclopidae, 1 exclusively with Harpacticoida. In 13 wells, i.e. 3 in the districts of Lokossa, Pobè, and Parakou and the 4 wells at Porto-Novo, representatives of the genus *Alloccyclops* were the only cyclopines found (Tables 1–4: exclusively dark grey background cells). All other wells accommodate a more diverse fauna with species of the genera *Afroccyclops*, *Thermocyclops* and *Mesocyclops* as the most frequently observed taxa. *Alloccyclops* species rarely co-occurred with another cyclopine in the districts of Parakou (3 of the 16 wells harbouring copepods) and of Lokossa (1 of 10 wells). In contrast, *Alloccyclops* was found living among other copepods in 5 of the 10 wells in the district of Pobè.

Co-occurrence of two species of *Alloccyclops* has been registered in 3 wells: *A. nudus* sp. n. and *A. pilosus* sp. n. in A1 and B6 (Parakou district) and *A. spinifer* sp. n. and *A. pilosus* sp. n. in well Pb6 (Pobè district).

A. pilosus sp. n. is the most widespread species. Reported from 16 wells, its presence in the northern district of the study area (Parakou) and the southern districts (Pobè, Porto-Novo, Kétou) spans the entire Ouémé drainage basin. *A. spinifer* sp. n. appears

to be restricted to the district of Pobè but was also detected in the occasionally visited well at Zogbodomé. It might be that the species has a preference to the southern and less elevated parts of central Bénin. Pobè and Zogbodomé are located in the Ouémé drainage basin. The occurrence of *A. nudus* sp. n. is restricted to the district of Parakou, in the northern and most elevated part of the Ouémé drainage basin. *Allocyclops sakiitii* sp. n. occurs only near Lokossa located in the drainage basin flooded by the rivers Mono and Couffo. The presence of the latter species in the occasionally visited well at Abomey-Calavi (located at the western limits of the Ouémé basin) is probably a result of the mixing of the waters from the Ouémé and Mono-Couffo basins in the coastal zone.

Subsoil characteristics

Bénin is well endowed with a dense river system flowing from north to south in central and southern Bénin (Ouémé and Mono-Couffo basins). Drainages in northern Bénin (roughly above 10° N) form tributaries of the large West-African rivers and run north-west (Volta basin) and north-east (Niger basin). Rivers are particularly subject of a flashy response to rainfall. They retain most of the year a low water level (large rivers) or disappear completely at the surface (many second rank tributaries). Villages have always been dependent on presence of a sufficient supply of groundwater during the long periods of drought for which human made hand-dug-wells and natural springs in the laterite soils have been the solution.

Laterite soils are subsoils of equatorial forests, the savannahs of humid tropical regions and of the Sahelian steppes (Tardy et al. 1991; Tardy 1997). They are a product of intensive weathering of the basement rocks and show distinct horizons. Their extension varies from 5 to 10 m below the ground level with the general translocation of clays towards the base (Bonsor et al. 2013). The clayish base layers support a primary aquifer with a regional extension. Depending on the local topography, a narrow to wide zone stretches between the laterite and the basement rock on which the deeper aquifer (vadose) develops (Alassane et al. 2010; MacDonald et al. 2012).

The hydrodynamic properties of a laterite aquifer are characterized by extreme variations with rapid lateral throughflow in the upper horizon during the wet season, and a narrow range of natural groundwater levels for much of the year. The majority of wells studied herein show this rather shallow to low water level (Tables 1–4: water-level fluctuation).

Members of the genus *Allocyclops* display a curious combination of conservative and advanced features. The combination of the large body size, the fully developed mouthparts and the complete armature compliment on the legs is in contrast with the general image of a subterranean cyclopine. The 11-segmented antennules and the 2-segmented rami of the legs however, are in accordance with a subterranean life style. It is assumed (Fiers 2012) that *Allocyclops* branched off directly from a epigene stock. The harsh and extreme variable environmental epigene circumstances may have been the main forces which favored the invasion of the subsoil aquifer by the ancestor.

Thus far, only *A. chappuisi* has been explicitly reported from a laterite spring (Kiefer 1932; Chappuis 1934). *A. cavicola* was discovered in a cave formed in a limestone outcrop of the laterite surroundings in Bas-Congo (Chappuis 1951; Leleup 1956). The poorly known *A. beadlei* was found in a marshy environment in Uganda (Lindberg 1956). The latter, clearly in a far state of decomposition, was possibly washed out of the laterite deck beds. With the addition of the four herein described species, it appears that the members of the genus *Alloccyclops* are particularly well adapted to live in the shallow and variable environment of laterite groundwater. The genus is, as far as conclusions can be drawn, a typical African sub-Saharan taxon (taking in consideration the temporary assignment of *A. botosaneanui*).

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Food sources of selected terrestrial cave arthropods

Jaroslav Smrž¹, Lubomír Kováč², Jaroslav Mikeš², Vladimír Šustr³,
Alena Lukešová³, Karel Tajovský², Alena Nováková³, Petra Režňáková¹

1 Department of Zoology, Faculty of Science, Charles University, Viničná 7, Prague 2, CZ - 128 44, Czech Republic **2** Institute of Biology and Ecology, Faculty of Science, P.J. Šafárik University, Moyzesova 11, SK-04654, Košice, Slovak Republic **3** Institute of Soil Biology, Biology Centre of the Academy of Sciences of the Czech Republic, Na sádkách 7, CZ - 370 05, České Budějovice, Czech Republic

Corresponding author: Jaroslav Smrž (smrz@cesnet.cz)

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Abstract

As caves represent an extreme biotope with limited food sources, one might expect cave animal communities to exhibit low feeding specialization and to consume generally whatever organic matter is available. To test this hypothesis, we studied the feeding habits of several arthropod species in Slovakian and Romanian caves. A microanatomical approach utilizing histological methods was selected for this study. While saprophagous animals dominated, our study revealed variability within this nutritional group. Preferences ranged from fungal propagules (for the millipede *Trachysphaera costata*) to bacteria on bat guano (for the oribatid mite *Pantelozetes cavaticus*) and to cyanobacteria (for the microwhip scorpion *Eukoenia spelaea*). The terrestrial isopod *Mesoniscus graniger* consumed a mixture of organic and inorganic substrates with plant material in various caves. These findings confirm an adaptability and phenoplasticity and, hence, a variability of characteristics developing under the pressure of extreme environmental factors.

Keywords

Caves, feeding habits, Isopoda, Diplopoda, Collembola, Acari

Introduction

Caves constitute an extreme or at least unique biotope in terms of their abiotic as well as biotic conditions (Seckbach 1999; Culver and Pipan 2009). Abiotically, they differ from epigeal habitats by their relatively low and stable temperature, poor or no light, and mostly high moisture (Vandel 1965). These conditions affect biotic factors such as food availability for the cave biota. Saprophagous, necrophagous, carnivorous and microorganism eaters (bacteriophagous, mycophagous and algivorous) can be all found in such an environment but their feeding habits have rarely been thoroughly studied. The diet of terrestrial cave arthropods has been predominantly studied in crickets (e.g. Lavoie et al. 2007; Di Russo et al. 2014) and beetles (e.g. Paoletti et al. 2011, 2013; Bradford et al. 2014). However, very limited data are available regarding the feeding habits of other cave arthropods, such as Acari, Isopoda, Diplopoda and Collembola.

We hypothesized that impoverishment of food sources in the cave environment results in poor consumer communities and probably in reduced specialization of nutritional niches. We should have expected, therefore, to find general consumption of whatever food is offered and without any conspicuous selection, as well as grazing of all available organic matter regardless of its palatability or digestibility. To test this hypothesis, we sampled representatives from several invertebrate groups in selected Slovakian and Romanian caves and then analyzed their gut content.

Material and methods

Nearly all animals were sampled individually in Ardovská Cave (south-eastern Slovakia), and only *Mesoniscus graniger* (Fruvaldszky, 1865) (Crustacea, Isopoda, Oniscidea) originated from Domica Cave (south-eastern Slovakia) and three Romanian caves (Ziditã, Mãgura, Fãnațe). The research adhered to the conditions of Licence # 3102/2009- 2.1/jam, from the Ministry of the Environment of the Slovak Republic, certificate of competency per Act No. 543/2002, and for Romania the authorization no. 340 of the Speleological Heritage Commission of the Ministry of Environment, Waters and Forests.

In details, representatives of several animal groups were collected for analysis of gut content:

– from Ardovská Cave:

- 16 specimens of microwhip scorpion *Eukoenia spelaea* (Peyerimhoff, 1902) (Arachnida, Palpigradi);
- 12 specimens of oribatid mites *Pantelozetes cavaticus* (Kunst, 1962) (Acari, Oribatida);
- 10 specimens of millipedes *Trachysphaera costata* (Waga, 1857) (Myriapoda, Diplopoda);
- 22 specimens of springtails, 10 of *Protaphorura armata* (Tullberg, 1869). and 12 of *Folsomia candida* Willem, 1902 (Hexapoda, Collembola).

– from Domic Cave and Romanian caves (Ziditã, Mãgura, Fãnaþe):

27 specimens of terrestrial isopods *Mesoniscus graniger* (Slovakia – 10 individuals, Romania – 17 individuals).

A microanatomical approach utilizing histological methods was selected for this study (Smrþ 2002). Animals were fixed in modified Bouin-DuBosque-Brasil fluid (Smrþ 1989) then embedded in Paraplast Plus (Fluka), sectioned on a Leica 2155 rotation microtome (0.005 mm thickness), and stained in Masson's trichrome. Occasionally, this was combined with observation using a Nomarski differential interference contrast prism (DIC). All sections were examined under an Olympus AX-70 microscope. The type and amount of gut contents (presence of food in all parts of gut or only in some ones), activity of the gut walls, and storage of nutrients (glycogen) inside the body were observed, according to the method described by Smrþ (2013).

Fresh specimens of *Eukoենenia spelaea* were viewed using a Leica TCS SP5 X confocal microscope with autofluorescence and red channel (emission spectrum 588–683 nm, excitation wavelengths 548–557–567 nm).

Plating of cave substrate on the cyanobacterial medium BBM in 1.7% agar plates was performed to confirm the cyanobacteria presence in Ardovská Cave.

Results

The results of gut contents in all tested species were the same inside each taxon.

Spherical cells (mostly 0.003–0.008 mm or larger) in the guts of paligrades (*Eukoենenia spelaea*) were determined to be cyanobacteria (Fig. 1). Under confocal microscopy, paligrade guts (4 specimens) were observed to be filled or crowded by spherical cells (Fig. 2). The digestibility of those cells was confirmed by very intensive deposition of glycogen in the gut diverticula. All sectioned microwhip scorpions (12 specimens) exhibited the same characteristics. The plating of cyanobacteria from cave substrate confirmed the presence of those cyanobacteria in Ardovská Cave. Unlike the cyanobacteria that had been swallowed, those plated had glycocalyx (Fig. 1, blue arrowhead).

Pantelozetes cavaticus mites, meanwhile, were characterized by a food bolus in the mesenteron, with several concentric membranes and many small bacterial cells between them (Fig. 3). Several specimens exhibited some particles of amorphous red mass in the bolus. The palatability of grazed food was linked to glycogen particles around the gut and a very intensive apocrine secretion in the blind projection of the mesenteron (the mesenteric caeca).

On the other hand, organic particles, and especially microfungus conidia, were grazed by the robust millipede *Trachysphaera costata* (Fig. 4). Those were mixed with wood fragments within an organic amorphous mass.

The observed springtails can be differentiated into two nutritional groups. In specimens of *Protaphorura armata* (Onychiuridae) the mesenteron contained a uniform mass of bacterial cells (Fig. 5), while in those of *Folsomia candida* (Isotomidae) a mixture of

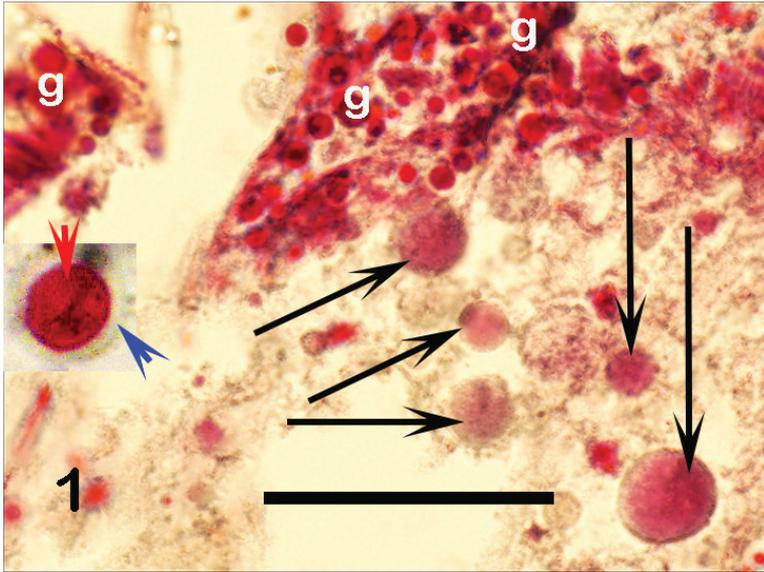


Figure 1. *Eukoenenia spelaea* – gut with food (large cells, indicated by black arrows). Red arrowhead = plated cyanobacteria in cave substrate (inset), blue arrowheads = glycocalyx. Stained with Masson's trichrome. Abbreviation: **g** glycogen deposits. Scale bar: 0.02 mm.

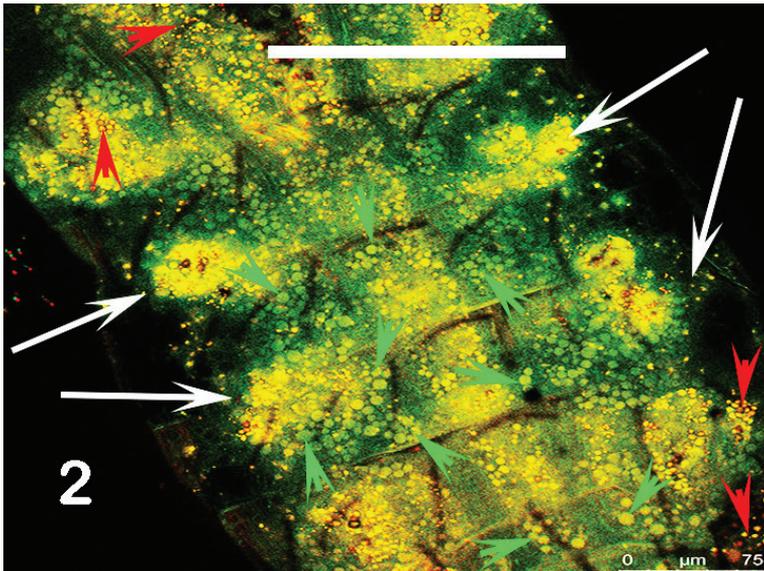


Figure 2. *Eukoenenia spelaea* – gut, confocal microscopy. Autofluorescence under red channel (emission 588–683 nm, excitation 548–557–567 nm). White arrows = gut diverticula, green arrowheads = cyanobacteria, red arrowheads = glycogen deposits. Scale bar: 0.1 mm.



Figure 3. *Pantelozetes cavaticus* – mesenteron with concentric bolus. Black arrowheads = apocrine secretion of the walls of mesenteron, red arrowhead = particle of amorphous mass in food bolus. Stained with Masson's trichrome. Abbreviations: **ba** bacteria loosely out of food bolus **fb** food bolus **g** glycogen deposits **go** gonads **me** cavity of mesenteron. Scale bar: 0.02 mm.



Figure 4. *Trachysphaera costata* – gut with microfungal conidia (arrows). Stained with Masson's trichrome. Scale bar: 0.02 mm.

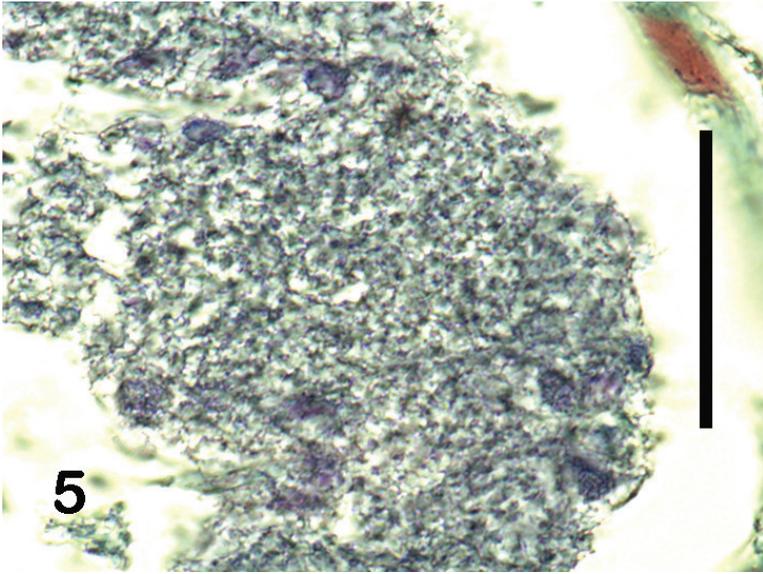


Figure 5. *Protaphorura armata* – gut with bacterial cluster. Stained with Masson's trichrome. Scale bar: 0.02 mm.



Figure 6. *Folsomia candida* – gut with microfungal conidia (arrows). Stained with Masson's trichrome. Scale bar: 0.02 mm

organic particles including microfungal conidia were found (Fig. 6). The digestibility of this substrate was confirmed by intensive apocrine secretion of the gut walls.

The terrestrial isopod *Mesoniscus graniger* was sampled in several Carpathian caves. We were thus able to compare the gut contents of individuals from distant localities.

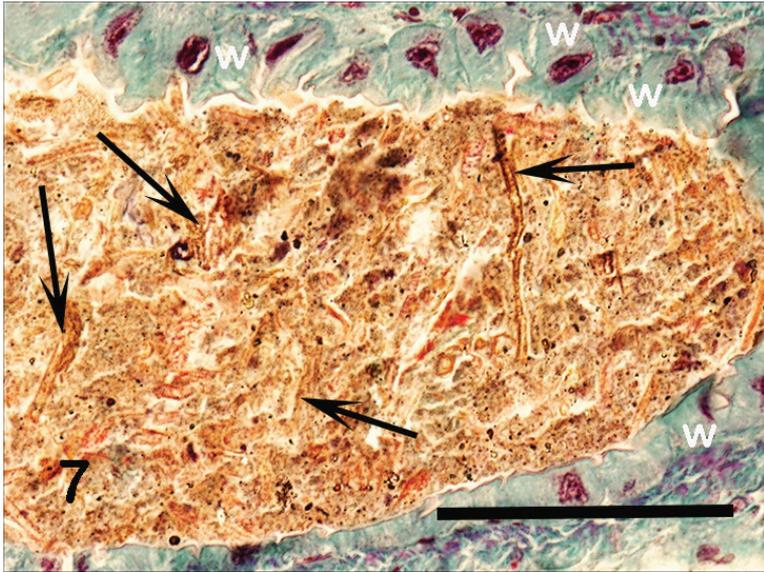


Figure 7. *Mesoniscus graniger*, Domica Cave, Slovakia – gut with food (plant remnants indicated by arrows). Stained with Masson's trichrome. Abbreviation: **w** gut walls with red nuclei. Scale bar: 0.05 mm.

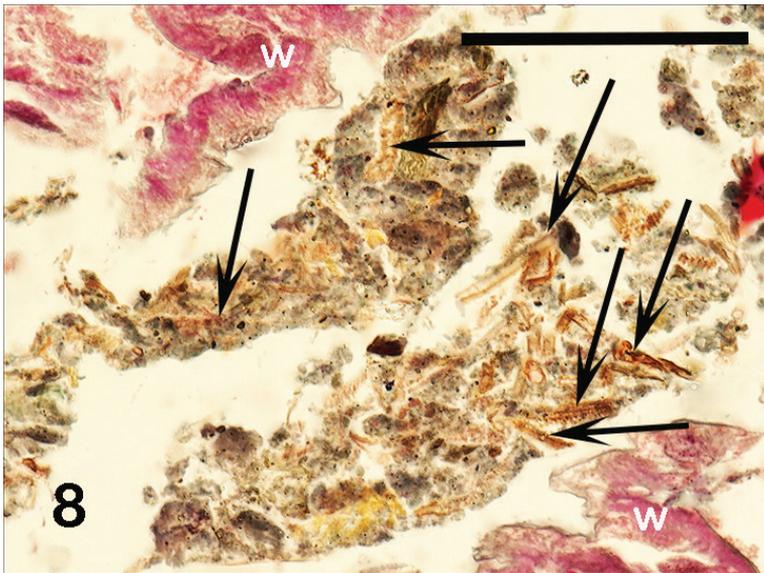


Figure 8. *Mesoniscus graniger*, Ziditã Cave, Romania – gut with food (plant remnants indicated by arrows). Stained with Masson's trichrome. Scale bar: 0.05 mm.

All were similar to one another regardless of the cave. The dominating mass was comprised of a mixture of organic and inorganic substrates with remnants of plants, both having no affinity for the used stain (Figs 7, 8). For the overwhelming majority of the analyzed animals, no fungal propagules were found.

Discussion

In spite of the limited food sources for cave-dwelling animal species, some nutritional specialization is evident. Food sources are used very thoroughly. First details of prey capture and cyanobacteria diet in palpigrades were published by Smrž et al. (2013). Cyanobacteria known for its ecological plasticity, with resistance against low or high temperatures, dryness, as well as poor light (Fay 1965) is able to grow also in caves.

The oribatid mite *Pantelozetes cavaticus* inhabited heaps of bat guano. The specimens in this study exhibited a concentric structure of food bolus in their mesentera. Such structure with bacterial cells between membranes confirms bacterial nutrition (Smrž 1989). Bacteria are a rather palatable and digestible food, as confirmed by glycogen deposition around the gut. The amorphous red mass in the bolus was clearly guano. The millipede *Trachysphaera costata* exhibited frequent consumption of fungi. Bacterial nutrition was demonstrated in the onychiurid springtails *Protaphorura armata*, while a selection of microfungal conidia were found in the gut of the isotomid *Folsomia candida* as confirmed by secretion activities in their gut walls. The food selection of the Collembola appears to be broad, reflecting various microhabitats inhabited by representatives of this group (Tebbe et al. 2006). *F. candida* and *Heteromurus nitidus*, frequent cave inhabitants, may even feed on nematodes (Lee and Widden 1996; Fiera 2014).

The isopod *Mesoniscus graniger* represents a contrasting case in nutrition selection, as seen in its low specificity of grazed food. Its gut was filled with mineral and organic material, with no indication of selection. Such condition was recorded in Domica Cave in Slovakia as well as in caves in Romania. On the other hand, that might be indicative of some consistency or even some type of selection in the feeding habit of this species. A wide range of food (fungi, algae, bat guano) was recorded in feeding experiments in Domica Cave, Slovakia (Šustr et al. 2005). However, a subsequently improved methodology that included more detailed laboratory feeding preference tests showed that *M. graniger* exhibits a clear preference for grazing on cave sediment and on laboratory cultures of some algae species over other species of cave algae and fungi (Nováková et al. 2005; Šustr et al. 2005). Both subspecies *M. graniger dragani* (Magura cave) and *M. graniger graniger* (Zidita and Fanate caves) had the same food in their guts. Nevertheless, more details can be found in small populations according to their different mouthparts and also provide more details of the food preferences (Giurginca, Šustr and Tajovský 2012).

While our hypothesis had assumed negligible selection, there is in fact conspicuous food selection by terrestrial arthropods in the studied cave biotopes. The limited food offer seems to be used very unambiguously and thoroughly by the invertebrate communities. Therefore, the competition for food can be actually regarded as very low.

Acknowledgments

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Spatial and temporal fluctuations of the abundance of Neotropical cave-dwelling moth *Hypena* sp. (Noctuidae, Lepidoptera) influenced by temperature and humidity

Rodrigo Lopes Ferreira^{1,2}, Vanessa Mendes Martins¹, Emanuelle Arantes Paixão¹,
Marconi Souza Silva^{1,2}

1 Programa de pós-graduação em Ecologia Aplicada, Departamento de Biologia, Universidade Federal de Lavras. C.P. 3037, CEP. 37200-000 Lavras, MG, Brasil **2** Centro de Estudos em Biologia Subterrânea, Departamento de Biologia/Setor de Zoologia Geral – Universidade Federal de Lavras. C.P. 3037, CEP. 37200-000 Lavras, MG, Brasil

Corresponding author: *Rodrigo Lopes Ferreira* (drops@dbi.ufla.br)

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Abstract

The present study evaluated the seasonal variation of a population of *Hypena* sp. in the Gruta Taboá (Sete Lagoas, Minas Gerais, Brazil), in relation to changes in temperature and humidity during the dry (July 1999 and July 2000) and rainy (January 2000 and January 2001) seasons. The *Hypena* sp. population responded to external seasonality, being distributed closer to the cave entrance during the rainy season, in which temperature and humidity fluctuated around 21 °C and 85%, respectively. During the dry season abundance was higher in sections farther from the entrance (deeper sections) (19.2 °C temperature and 80% humidity). The results showed that this species is influenced by external environmental factors, even in a tropical region where the external climate fluctuations are lower compared to temperate regions.

Keywords

Noctuidae, *Hypena*, cave, seasonal variations, temperature, humidity, Neotropical

Introduction

Caves, in general, show little variation of environmental parameters such as temperature and humidity compared to the external environment. Thus, they are often considered more stable than the surrounding external environments (Poulson and White 1969, Howarth 1980, 1993).

According to Michell (1969), tropical caves are climatically more stable compared to temperate caves. However, the environmental conditions are not homogeneous throughout the cave, being composed by a series of microclimates which vary according to the cave area (Romero 2009). Characteristics like dimensions, width and number of cave entrances interfere on environmental parameters (Ferreira 2004).

Among the environmental parameters that influence the cave fauna is temperature, which is held as one of the main determinants of metabolic activities (Gillooly et al. 2001, 2002) and life history of organisms (Willott and Hassall 1998, Hodkinson et al. 1999). Parameters such as humidity and rainfall can also affect the ecology of several species (Rodrigues 2004).

In tropical regions, the seasonal fluctuations in the abundance and richness of insects are notorious and arise from the variation of temperature, humidity and rainfall (Wolda 1978). Such seasonal changes require specific strategies of the organisms that allow them to live under this variable conditions. Insects utilize many adaptations to avoid desiccation, including reduction of surface area and deposition of waxes and fat in the epicuticle, besides seeking for more stable environments or microhabitats with high humidity (Villani et al. 1999).

Many studies showed the effects of external climatic fluctuations on populations or subterranean communities in the temperate region (Bourne 1976, Bourne and Cherix 1978, Carchini et al. 1994, Novak et al. 2004, Tobin et al. 2013). These effects on population dynamics are farther expected in geographical regions where external climatic fluctuations are more intense (Michell 1969). However, modifications on cave population dynamics due to fluctuations of the external environmental parameters have been neglected in tropical regions, perhaps due to the false impression that both small temperature and humidity fluctuations (typical of tropical regions) have little or no effect on the subterranean fauna.

In this context, the present study aimed to determine seasonal variations in the spatial distribution and abundance of the population of the troglaxene moth *Hypena* sp. (Noctuidae, Lepidoptera) in a limestone cave in Southeastern Brazil.

Methodology

Study area

The study was conducted in a limestone cave with approximately 1000 meters of linear extension, called Taboa cave (19°28'30.0"S, 44°19'41.7"W – DMS (degrees, minutes,

seconds), located in the municipality of Sete Lagoas, Minas Gerais, Brazil. This cave is located in the Cerrado biome (Brazilian Savannah), however, the area surrounding the cave is quite altered, and much of the original vegetation has been removed for the establishment of pastures (Figure 1A). The climate classification is Cwa (Climate Warm or Humid Subtropical Climate), savanna climate with dry winter and humid summer with rain (Álvares et al. 2013). The average annual temperature in the municipality where the cave is located is 22.1 °C and the relative humidity varies around 70.5%. The average annual rainfall is 1,340 mm, with December as the rainiest month and August, the driest (Sans 1986).

Taboa cave comprises a vadose system and its main conduit is crossed (half of its extension) by a subterranean stream that enters the cave in its deepest part. The distal part of the cave is rather moist, and food resources include plant debris brought in by the water and especially bat guano. There is a small aperture (45×60 cm) in this distal part, which can not be used by humans due to the siltation which have strongly reduced the natural entrance. However, it can be used as an entrance for many invertebrates, although only few specimens of *Hypena* sp. (less than 10) were found in this area.

The area near the main entrance (which is also small – 1.5 m high and 2 m wide) is quite dry throughout the year (Figure 1). Most of the *Hypena* sp. population lives near the main entrance (Figure 1) and the present study focused on this area.

Methods

The study was conducted within the first 120 m from the cave entrance. This part included the area where individuals of *Hypena* sp. were regularly found throughout the study. After the first 105 meters from the main entrance, specimens were eventually observed. These individuals observed after 105 meter from the main entrance were not considered in this study, since they were probably under the influence of the second opening, more than 650 meters distant from the main entrance of the cave. The first part of the cave was divided into five-meters sectors from the entrance to evaluate the temporal and spatial variations of *Hypena* sp. correlated to the seasonal variation of both temperature and humidity. Sampling was performed in the peaks of the dry periods (July 1999 and July 2000) and the peaks of the rainy periods (January 2000 and January 2001). The temperature and humidity measurements were taken in each sector once, every sampling period, by a digital thermohygrometer (Hygrotherm Oregon Scientific). The device was positioned on the cave floor and during the measurements the researcher stayed far from the device to prevent eventual influences on the cave atmosphere. *Hypena* sp. specimens were recorded in each sector and their distribution along the conduit was plotted on a cave sketch.

The surface temperatures and precipitation were obtained by consulting data from the National Institute of Meteorology (INMET), Sete Lagoas station, 16 km from the cave.



Figure 1. **A** Photograph indicating the cave entrance and the surrounding region, whose native forest was turned into pasture **B** Conduit located in the area near the entrance **C** Individuals of *Hypena* sp. resting on the cave wall.

Data analysis

The analysis of variance (ANOVA) was performed by using the R software in order to determine whether there were differences in abundance between sampling periods.

Since the data corresponds to a set of points that indicate the spatial location of each sampled individual inside the cave, we used Spatial Point Pattern Analysis (Diggle 2013, Bailey and Gatrell 1995, Gatrell et al. 1996). This analysis allowed to investigate the spatial relationships between individual occurrence points for each monitoring to identify spatial distribution patterns. The first step of this spatial analysis is to make a dot map. This enables an initial visualization of both the shape of the study area and the possible spatial pattern of the analyzed data (Bailey and Gatrell 1995). The spatial distribution patterns may be independent (completely random), regular or clustered. The next step is to evaluate the intensity of the point pattern. It corresponds to the average density of points, i.e., the expected number of points per unit area (Baddeley 2010). This evaluation allows describing how the expected value and mean vary across the space. Again, it is possible to identify by observing the intensity maps the existence of a possible spatial pattern and also if there are sectors in which individuals tend to cluster. The estimation of intensity values were made using an Isotropic Gaussian Kernel (Baddeley 2010, Gatrell et al. 1996). Subsequently, estimations of the K function together with simulation envelopes by the Monte Carlo method with 5,000 simulations (Baddeley 2010, Gatrell et al. 1996) were conducted to identify statistically the spatial patterns. According to Ripley (1981) the significance level of 5,000 simulations is about 0.0004. All analyses were performed in R software, Spatstat package (Baddeley and Turner 2005).

Results

Temporal variations of environmental parameters

The measured temperature in the studied cave stretch ranged from 18.6 °C to 23.1 °C. In the same period, the measured air relative humidity ranged from 50% to 96%.

During the dry periods the environmental parameters (humidity, precipitation and temperature) at the surface were relatively similar in the two sampling years. July 1999 showed no precipitation, the temperature fluctuated from 13 °C to 27 °C and the air relative air humidity was 60% (INMET 2008). July 2000 presented temperature varying from 12 °C to 26 °C and air relative humidity was 59.7% and there were 8.9 mm of precipitation (INMET 2008).

In January 2000 the temperature oscillated between 19.4 °C and 29.2 °C, with 74% air relative humidity and 387 mm of rainfall. In 2001, the same month had temperature, air relative humidity and precipitation of 18.8 °C–30.1 °C, 68.3% and 105.5 mm, respectively (INMET 2008).

The highest variation of temperature and humidity in the cave was observed near the entrance in both sampling periods (dry and rainy). As the distance from the entrance increased, there was an increase in humidity and a decrease in temperature until approximately halfway into the sampling area (70 m). However, in the final part of the study area the temperature tended to stabilize, a pattern that remained similar during the study (Figure 2A).

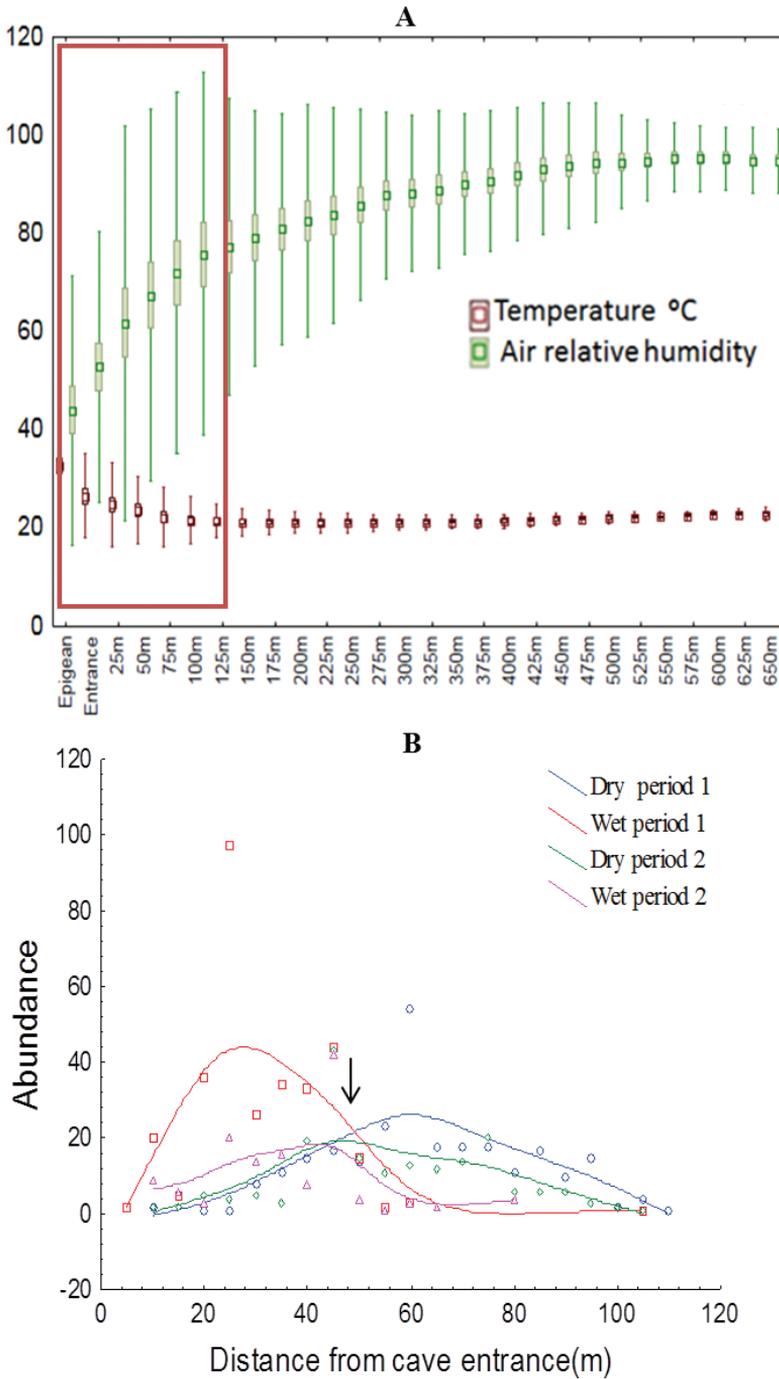


Figure 2. A Variation in temperature and humidity along the Taboa cave, showing a tendency to stabilize in the deeper parts of the cave. The table shows the section in which the *Hypena* sp specimens were collected (**B**) Change in abundance over the transects, the arrow indicates the spatial extent where the effects of the surface seasonality promote decrease and expansion in the population distribution.

Both temperature and humidity in the cave ranged in July 1999 (dry period), from 22.7 °C to 18.7 °C and 84–50%, respectively. In the following year (July 2000) the temperature and humidity ranged from 20.6 °C to 18.6 °C and 67 to 92%, respectively. The rainy season of 2000 (January) presented temperatures ranging from 23.7 °C to 20.5 °C and relative humidity from 71 to 91%. In the following year, the temperature and humidity ranged from 25 °C to 20.3 °C and 66–96%, respectively.

Temporal variations in the abundance of *Hypena* sp.

In the first visit to the cave (during the dry season – July, 1999) we observed 260 individuals of *Hypena* sp. while in the second visit (rainy season – January, 2000) we found 318 individuals. In the third visit, conducted in July, 2000 (dry season), we observed 192 individuals, and in the last visit (January, 2001 – rainy season) we observed 132 individuals. Accordingly, a total of 452 individuals of *Hypena* sp. were observed in the dry seasons and 450 during the rainy seasons. There were no significant differences in the number of individuals between the dry and rainy seasons ($F = 0.522$, $df = 54$, $P = 0.6645$). *Hypena* sp. individuals occurred in a temperature range between 18.6 °C and 25 °C and air relative humidity between 50 and 96%. Out of these intervals, no individuals were observed.

During the dry periods individuals were more abundant in the cave part where the temperature and humidity were around 19.2 °C and 80%, respectively, showing a preference for the areas farther inside the cave in this period. However, during the rainy seasons the organisms preferentially occupied areas closer to the entrance, where the temperature and humidity fluctuated around 21 °C and 85%, respectively.

During the rainy seasons, peaks of higher abundance were observed around 20 to 40 meters from the cave entrance. The peaks of abundance in the dry periods occurred slightly deeper inside the cave, around 50 to 70 meters from the cave entrance (Figure 2B).

Spatial analysis

Figure 3 summarizes the results obtained from the Spatial Point Pattern Analysis of the second visit to the cave (January 2000). In the dot map (Figure 3A) is the shape of the study area to see the distribution of the analyzed points. The observed individuals tend to concentrate in the innermost cave region, which possibly would feature a clustered pattern. This statement is confirmed by the K function estimations (Figure 3B), which allowed to identifying the spatial pattern of the data points. The theoretical K function refers to the expected values of this function assuming that points are completely random. The Monte Carlo method allowed to estimate the value upper and lower envelopes of the simulated K-functions defining the shaded region. This region corresponds to the completely random pattern. However, the empirical K function, which is estimated based on observed data, lies outside of this region indicating that the

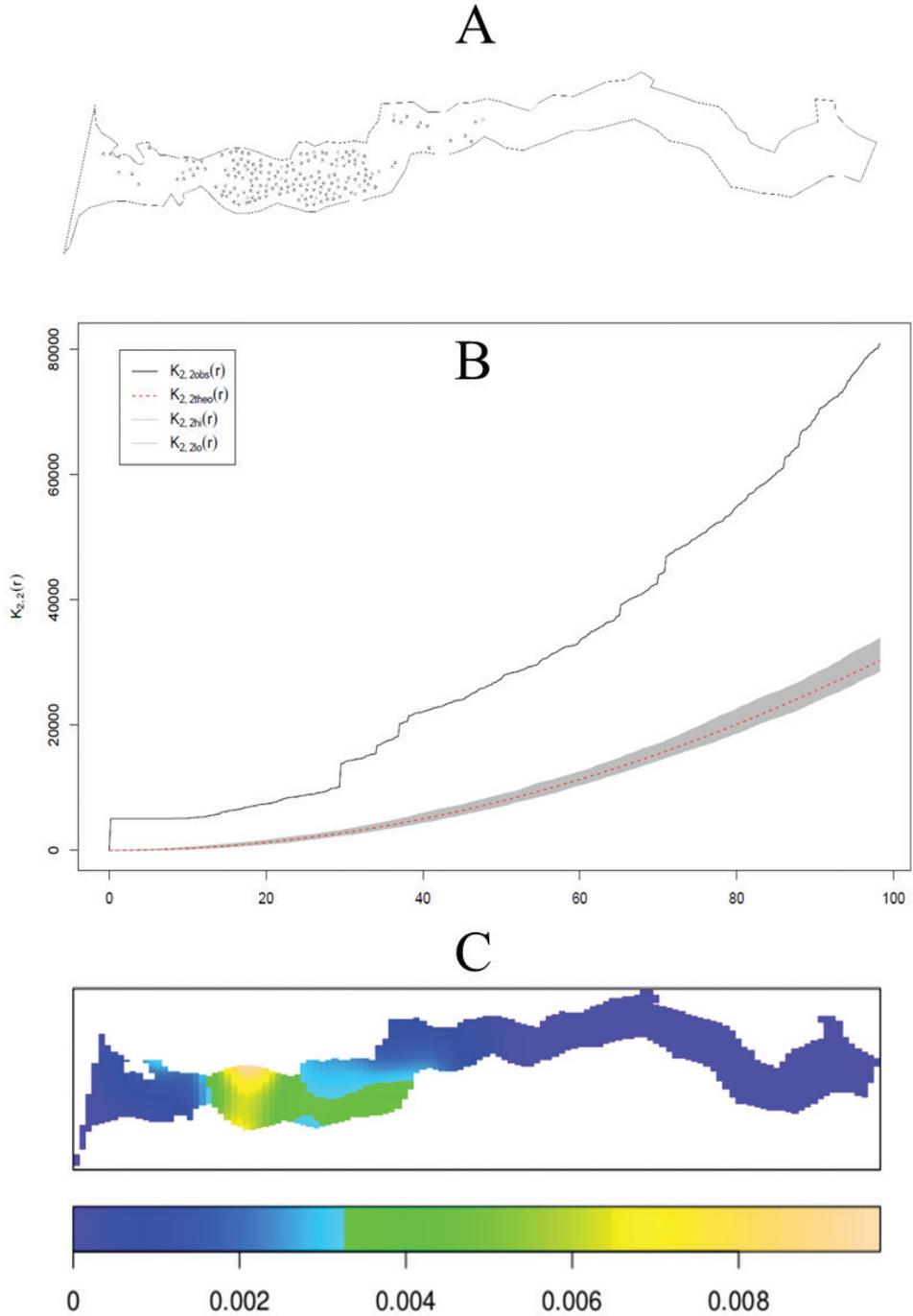


Figure 3. Spatial Point Pattern Analysis of the second monitoring (January 2000). **A** Dot map and **(B)**, shows the estimations of the function $K(r)$ (r is the distance argument, Dashed line corresponds to the theoretical value of this function is Complete Spatial Randomness and solid lines the Observed value of the K function for the date pattern) **C** Map Kernel Estimates of intensity.

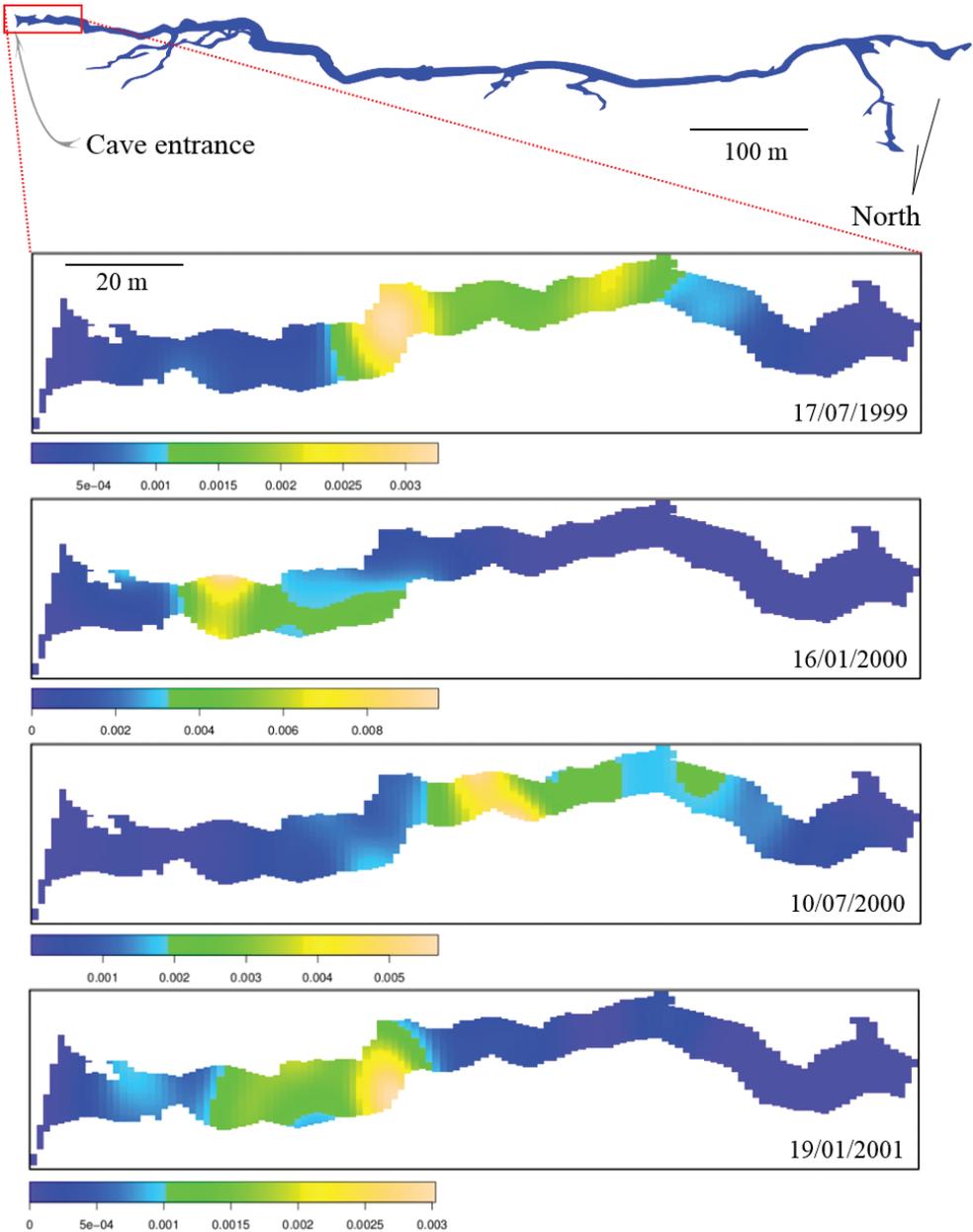


Figure 4. Spatial distribution maps of *Hypena* sp. demonstrating different densities between seasons. In the dry seasons (17/07/1999 and 10/07/2000) individuals are located in the deepest region of the cave, an opposite pattern during rainy seasons (16/01/2000 and 19/01/2001) when the population of individuals becomes denser in the region near the cave entrance. Blue colors indicate low densities while light yellow colors indicate high densities.

analyzed pattern is not random. Moreover, the values of this function are higher than those of the theoretical estimates, which characterize a clustered pattern. This pattern can also be seen in the intensity map (Figure 3C) in which regions with higher average density of points are observed. The clustered pattern observed in second sampling period (January 2000) has also been proven for all the other three sampling periods. The cluster spatial distribution has varied as follows: in July 1999 and July 2000, the sampled individuals concentrated in the innermost cave region, and in January 2000 and January 2001, clusters were observed in the region closer to the cave entrance (Figure 4).

Discussion

Both, the temperature and humidity varied between sampling periods and between the different cave sectors. However, the highest variations were observed near the entrance, where *Hypena* sp. preferentially occurred and presented the higher abundance. Although it is a troglone species and capable to support variable conditions the population has modified its spatial pattern of distribution, according to the variations of the external environment. The organisms tended to be distributed preferably in the regions farther from the entrance in the drier periods and in regions closer to the entrance in the wettest periods.

Caves have a tendency towards stability but cannot be characterized as closed systems (Bourgues et al. 2006), considering that during the winter the caves experience heat and humidity loss, countering the heat and humidity gain in the summer (Freitas 2010). In an environment that tends towards climate stability it is likely that even small variations can lead to alterations in species abundance or distribution (Tobin et al. 2013). Thus, the abiotic factors in a cave can also determine the local faunal distribution, especially considering insects, as observed in the present study.

The cave climate changes due to variations at the surface. During the cold winter, dry air enters the cave that in turn influences the desiccation of the organisms which are in direct contact with the air stream (Tuttle and Stevenson 2011). Evaporation is determined by the relative humidity and under high humidity the evaporation is low. However, if the relative humidity changes from 99.5% to 99%, the evaporation rate will double (Buecher 1999), what brings drastic physiological consequences to the fauna (Singh et al. 2009). Thus, it is plausible to assume that in order to avoid desiccation during the winter, specimens of *Hypena* sp. migrated to the inner regions of the cave, in search of higher humidity.

According to Bourne and Cherix (1978), *Triphosa dubitata* have a preference for environments where temperature and humidity are higher (>11 °C and 80%). The authors also noted that in response to seasonal changes of the air flow inside the cave, *T. dubitata* migrated in the winter to the inner regions to avoid low temperatures and desiccation.

Bourne (1976) in a study carried out in the La Scierie cave (France) showed that five species, among them two Lepidoptera (*Thiposa dubitata* and *T. sabaudiata*), mi-

grated during the autumn and winter to the inner regions of the cave. The previous studies reinforce the notoriety of the spatial variation of temporary cave fauna in temperate climates as a result of external changes. However, works in tropical regions showing a similar pattern are virtually non-existent.

According to Wolda (1978), in the tropics, the temperature variation is generally low and therefore the variables that best explain the seasonality of insects are humidity and precipitation. Although not carried out with a Lepidoptera, an interesting study performed by Carchini et al. (1994) showed seasonal variations in a cricket population of *Dolichopoda geniculata* in Valmarino Cave (Italy). It was observed that during summer the inner region of the cave presented a higher density of organisms, and during winter the population occupied the outmost area. This pattern was the opposite of the observed in the present study, but it should be noted that the cited work was performed under the Mediterranean climate (dry summer and rainy winter). Therefore, this fact suggests that humidity can exert great influence on the seasonality of these organisms, since the two studies previously mentioned (Bourne 1976, Carchini et al. 1994) showed that in the higher humidity period (rainy) the two populations of different species (from distinct groups) preferred the cave entrance area. McKillop (1993), studying caves in Canada, found moths (*Triphosa haesitata*) only in caves whose humidity was above 84%, leading to the belief that moisture is a limiting factor for the occurrence of this species.

Many species enter caves to avoid extreme temperatures (heat and cold) in order to establish themselves in an ideal or milder microclimate (Camp et al. 2007, Chelini et al. 2011). *Hypena* sp. showed a preference for a specific temperature and humidity range, and according to alterations of environmental parameters along the dry and rainy seasons, the population altered its distribution within the cave.

Although the tropical climate oscillates less than the temperate climate and supposedly there is less influence of these external climatic variations on the subterranean fauna, this study demonstrated that even small environmental fluctuations change the population dynamics of a species inhabiting caves. Therefore, we emphasize the importance of further studies, since most of the works in the tropics do not describe seasonal variation of environmental factors in caves, not even the response of organisms to these changes. Our results highlight the importance of monitoring population dynamics of cave invertebrates in relation to environmental conditions.

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Spontaneous behavior of basal Copionodontinae cave catfishes from Brazil (Teleostei, Siluriformes, Trichomycteridae)

Bianca Rantin¹, Maria Bichuette¹

¹ Universidade Federal de São Carlos (UFSCar), Departamento de Ecologia e Biologia Evolutiva (DEBE), Rod. Washington Luís, km 235, Caixa Postal: 676, São Carlos, São Paulo, Brasil

Corresponding author: Bianca Rantin (bianca.rantin@gmail.com)

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Abstract

Cave animals are particularly interesting due to their behavioral specializations, resultant from evolution in isolation. We present data from a spontaneous behavior study (spatial distribution and preference for microhabitats) of two troglobitic catfish from Brazil: *Glaphyropoma spinosum* and a new species of *Copionodon*. We compared the data with those obtained of a sympatric epigeic species, *Copionodon pecten*. These Trichomycteridae species belong to a basal and apparently monophyletic subfamily – Copionodontinae, endemic to Chapada Diamantina, central Bahia state, eastern Brazil. We observed the fishes in natural and laboratory conditions through *ad libitum* and focal animal methods. Each spatial behavioral category (hidden, bottom, midwater, surface and wall swimming and stationary in the bottom) was timed individually, with a sample of 12 specimens per species. Unlike most troglobitic fishes, cave copionodontines tested herein did not extend exploratory behavior to midwater, with benthonic and thigmotactic-related exploratory behavior. This behavior is possibly related to its feeding behavior specializations, strong territorialism and photophobic behavior. The epigeic *Copionodon* species is also benthonic. The spatial behavior of the cave Copionodontinae could be interpreted as a retained and plesiomorphic character-state in relation to other trichomycterid catfishes.

Keywords

Spontaneous behavior, subterranean environment, Neotropical region, *Glaphyropoma*, *Copionodon*

Introduction

Stygobites are organisms restricted to the subterranean aquatic environment, which had evolved isolated in a peculiar selective regime, distinct from that of their ancestors: total absence of light, a tendency to environmental stability, lack of primary production and low energy intake (Culver 1982, Poulson and Lavoie 2000, Culver and Pipan 2009). In order to survive and effectively colonize the hypogean realm, subterranean organisms must reproduce, defend their territories and find food and mates in this environment, regardless of vision (e.g. Trajano 1989, Wilkens 1992, Moore and Sullivan 1997, Soares and Niemiller 2013). Several specializations related to life in the subterranean environment have been reported in literature - autapomorphies, called troglomorphisms (Arnold 1994).

These cave specializations include morphological troglomorphisms (for instance, reduction of eyes and melanic pigmentation and elongation of body and appendices) and behavioral ones (such as increase of midwater activity and reduction of photophobia, cryptobiotic behavior and circadian rhythms) (Trajano 2003). Among behavioral specializations, the spontaneous behavior is well studied for Brazilian troglobite fish (e.g. Trajano 1989; Trajano and Bockmann 1999; Bichuette and Trajano 2005; Bichuette 2003). Spontaneous behavior encompasses use of space, i.e., preference for microhabitats, spatial distribution (bottom, midwater or surface), burrowing, hiding and other behavior expressed in the absence of stimuli.

Considering cave fish with putative bottom-dwelling ancestors, living in an environment characterized by food scarcity, such as caves, it is beneficial to extend the spatial distribution, since it means increasing the probability of finding food in conditions of low prey densities, especially if associated with low predation rates and low competition (Trajano 1989). Thus, increasing midwater activity, as observed in catfishes and balitorids (Parzefall and Trajano 2010), could be considered adaptive (Trajano 2003).

In turn, the reduction of cryptobiotic behavior, namely, decreasing the hiding behavior, may be explained by the absence of visually guided predation in the hypogean realm: the pressure for maintaining the hiding behavior would be relaxed, and the character would regress (Wilkens 1992). In this case, reduction of behavior traits could be analogous to regression of morphologic ones, such as eyes and pigmentation, and probably correspond to characters that became neutral in subterranean habitat (Wilkens 1992; Culver and Wilkens 2000; Trajano 2003).

Most troglotic fishes belong to epigeal taxa which show cryptobiotic behavior, and there is a general tendency among such troglotic species towards reduction of cryptobiotic behavior along with an increase of midwater activity (Parzefall and Trajano 2010). Among Brazilian troglotic fishes we observed this tendency for Heptapteridae catfishes *Pimelodella kronei* Miranda Ribeiro 1907 (Trajano 1989), *Rhamdiopsis* sp. from Toca do Gonçalves, Campo Formoso (= *Taunayia* sp.) and *Rhamdiopsis krugi* Bockmann and Castro 2010 (formerly cited as *Rhamdiopsis* sp. from Chapada Diamantina) (Parzefall and Trajano 2010).

Brazil has 25 species of troglobitic fishes, most of them belonging to the siluriforme families – Callichthyidae, Heptapteridae, Loricariidae and Trichomycteridae (Proudlove 2010, Trajano and Bichuette 2010). Among Trichomycteridae, there are two troglobitic representatives of the Copionodontinae subfamily, both in caves from Bahia State – *Glaphyropoma spinosum* Bichuette, Pinna and Trajano 2008 and a new species undescribed of *Copionodon*. Copionodontinae is a basal taxon within the Trichomycteridae, lacking many synapomorphies (that is, characters shared by two or more taxonomic groups and derived from a common ancestral species) previously used to diagnose the family (Pinna 1992). It is probably a monophyletic group, diagnosed by several unambiguous synapomorphies, including a unique dentition composed of spatulate teeth not seen anywhere else in Trichomycteridae (Bichuette et al. 2008). They are represented by five species in two genera, *Glaphyropoma* and *Copionodon* (Pinna 1992, Bichuette et al. 2008)

Glaphyropoma spinosum and *Copionodon* new species are syntopic and coexist in sandstone/quartzitic caves. Cases of cave fish coexistence are rare in global terms and present an excellent opportunity to compare behavioral studies, since intraspecific competition for space, feeding and/or mating territory may occur (Trajano et al. 2002). These two species were studied with focus on feeding and agonistic behavior and reaction to mechanical stimuli, comparing to a sympatric epigeal species, *Copionodon pecten*. A previous study of phototactic behavior showed that *Glaphyropoma spinosum* is highly photophobic, even at low intensities (ranging from 40 up to 1,700 lux), while *Copionodon* sp. n. is photophobic under low light intensities (40 and 170 lux) and indifferent to high intensity (1,700 lux). The epigeal related species, *Copionodon pecten*, showed indifference to light, even at high ones (Rantin and Bichuette 2013).

The comparison of the spontaneous behavior of Copionodontinae catfishes occurring in distinct habitats (subterranean and epigeal typical headwaters streams) will bring more evidence to understand the possible modifications related to the adoption of a subterranean way of life.

In order to understand whether the observed spontaneous behavior consist of specialization traits for Copionodontinae, we considered its basal phylogenetic position within Trichomycteridae clade. Therefore, we established the following question: Do the cave copionodontine *Glaphyropoma spinosum* and *Copionodon* sp. n. show reduction of cryptobiotic behavior and/or extended spatial exploratory behavior?

Materials and methods

Study area

Copionodontine catfishes are endemic to Paraguaçu river basin, in Chapada Diamantina, situated in central Bahia, eastern Brazil (Fig. 1). The climate is tropical semi-arid, with distinct wet/dry periods, Aw category (tropical wet and dry) according to Köp-

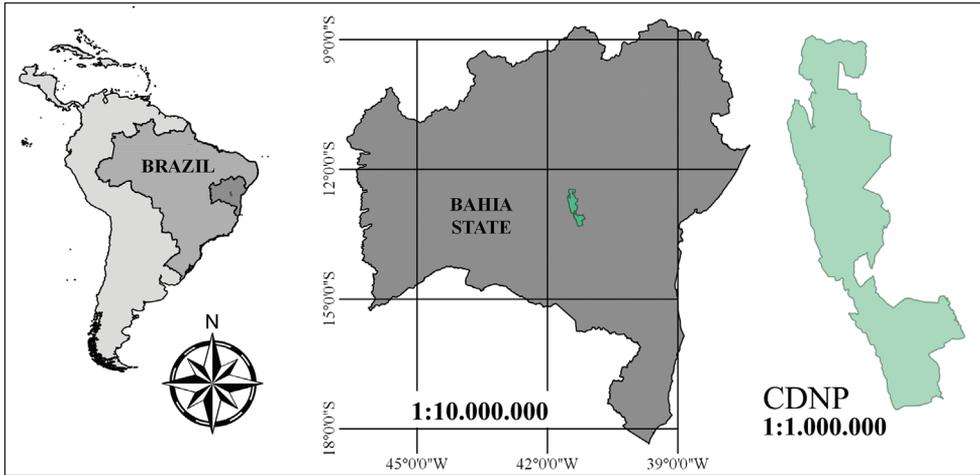


Figure 1. Copionodontinae occurrence area. Map of Brazil, indicating the Chapada Diamantina region, endemic area of Copionodontinae catfishes. In green, the Chapada Diamantina National Park (CDNP), eastern Brazil. Author: Diego Monteiro Von Schimonsky.

pen's (1948) classification. Rainy season is from November to March/April and the dry season between May and September (Brasil 1981).

The caves where subterranean copionodontines coexist are formed mainly by sandstone and quartzite rocks and are situated in the surroundings of Igatu village, within the limites of Chapada Diamantina National Park (CDNP) and are, therefore, protected by law. Specimens occupy waters with slow current, in pools and/or runs of subterranean streams (Fig. 2a, b). Apparently, the subterranean streams from Igatu do not have any connection with epigeian rivers.

The epigeian copionodontine, *C. pecten*, inhabits streams with interconnected pools of dark waters, with a high amount of organic matter, with sand, boulders and bedrock in the bottom (Fig. 2c).

Studied species

Glaphyropoma spinosum catfish show little intraspecific morphological variation: its cutaneous melanic pigmentation is homogeneous, light yellow to pale pinkish; the eyes are reduced and, in some individuals, covered by skin and not externally visible (Fig. 3).

Copionodon sp. n., on the contrary, presents a high degree of intraspecific variability in morphological traits: the degree of melanic pigmentation varies from pale gray to dark gray, sometimes with lateral and dorsal stripes. Eyes may vary from reduced and covered by skin to developed eyes as observed for epigeian copionodontines. Occasionally, the left or the right eye is larger than the other, showing an individual asymmetry in this character-state (Fig. 4).



Figure 2. Spatial behavior and natural habitats of copionodontine catfishes from Chapada Diamantina **a** *Glaphyropoma spinosum* (black arrow) swimming close to the bottom in a pool (Photography: Maria Elina Bichuette) **b** small stream in the cave environment, place where *Copionodon* sp. n. occurs (Photography: Jonas Eduardo Gallão) **c** natural pool where *Copionodon pecten* forages under the day-light (Photography: Bianca Rantin).



Figure 3. *Glaphyropoma spinosum* – fixed specimen, left lateral view and dorsal view (Photography: Pedro Pereira Rizzato). Standard length: 47.6 mm.



Figure 4. *Copionodon* sp. n. – fixed specimen, left lateral view and dorsal view (Photography: Pedro Pereira Rizzato). Standard length: 38.9 mm.

The epigeic species, *Copionodon pecten*, is clearly homogeneous in morphological traits (for morphological details see Pinna 1992): body is grayish in color, with iridescent spots and conspicuous lateral stripes; eyes well developed (Fig. 5).



Figure 5. *Copionodon pecten* – fixed specimen, left lateral view and dorsal view (Photography: Pedro Pereira Rizzato). Standard length: 43.2 mm.

Specimens collecting and laboratory maintenance

Copionodontinae catfish were hand-netted and transported in 3-liter containers, filled with water from the place of collection. The containers were conditioned in thermal boxes, with constant aeration, and low temperature was achieved with addition of recycled hypergel.

Intraspecific groups up to 10 individuals were maintained in 50-liter aquaria with continuous filtration, aeration and available shelters, made with sandstone rocks and big enough to fit the whole body. Aquaria were installed at the Laboratório de Estudos Subterrâneos (LES) at the Departamento de Ecologia e Biologia Evolutiva of Universidade Federal de São Carlos (UFSCar). Room conditions were manipulated to simulate aspects of cave natural environment, in order to maximize welfare and survival: it remained in permanent darkness (except during maintenance activities), water temperature was stabilized at 27 °C with air conditioning and pH was maintained near to natural habitat using rocks brought from the collection site. Epigeic Copionodontinae were also maintained in the LES, but in a separate compartment, covered with a black shield, where a lamp attached to a timer simulated the daily light cycle (12:12 light/dark hours). The catfish were fed once a week with commercial food for carnivorous fishes (dry or frozen *Artemia salina*) at no fixed days and times.

Animal rights

There are no specifications regarding behavior research in Brazilian Animal Ethics Law (11.794/2008). However, our financial support (Fundação de Amparo à Pesquisa no Estado de São Paulo (FAPESP - a public sponsoring agency) approved and supported our research (processes numbers 2008/08910-8 and 2008/05678-7). We also have a collection and transportation permission (license number 20165-1), provided by the Brazilian environment governmental agency ICMBIO (Instituto Chico Mendes de Biodiversidade). Furthermore, experiments were delineated prior to collection, in order to maximize the scientific value of individuals captured: reaction to mechanical stimuli, feeding, spontaneous and phototactic behavior (Rantin and Bichuette 2013) and were also assessed.

Field observations

The study of behavior in natural environment was performed by direct observations in four field trips: May 2009, August 2009, May/June 2010 and August 2010 (Fig. 2c), with a total of 40 hours. Behavioral categories exhibited in natural habitat were posteriorly compared with laboratory observations.

In the field, the focal-animal-method was used when individuals were found isolated in pools. Whenever in groups, fish were observed by *ad libitum* mode (Altmann 1974). Their behavior was classified into categories, in order to glimpse the species general pattern behavior in nature. These behavioral categories were based in protocols proposed in other studies (e.g., Buck 2000): A. hidden (hiding behavior on under blocks and striped pebbles), B, bottom swim (swimming with ventral region touching substrate); C, midwater swim; D, surface swim and E, stationary (at the bottom). Additionally, we observed gregarious (group formation) and solitary behavior.

Environmental physical characteristics were also recorded: fast or slow water current; bottom formed by silt, sand, gravel, pebbles, rock boulders and/or blocks. Studied individuals were observed/captured on/from the following localities: Parede Vermelha cave, Rio dos Pombos cave, Torras cave, Morro de Alvo cave, Coisa Boa river and Xavier river.

Spontaneous behavior analysis

Spontaneous behavior tests were performed a month after the fish collections. Twelve adult specimens from each studied species were tested individually: the troglobites – *Glaphyropoma spinosum* and *Copionodon* sp. n.; the epigean – *Copionodon pecten*.

Each specimen was placed in a 50 l test-aquarium, with a single den, with no filtering or aeration systems. After one hour of acclimatization, we started the tests,

always between 8:00 am and 6:00 pm. The observations were conducted under the same luminosity, for one hour. Considering that the epigeal species is indifferent to light, and that both cave species react the same towards low light intensities (both are photophobic), a 140 *lux* light intensity was used (enough for visualization of behavioral units) for the tests.

The behavioral categories were divided as follows: A. hidden (at least the anterior part of the body covert, inside den, with no attempts to dig the substrate), B. bottom swim, C. midwater swim (swimming in water column), D. wall swim (ventral region touching walls), E. surface swim and F. stationary (in the bottom, outside of the den). Swimming in water column was separated from swimming in wall due to field observations: these animals are used to swim close a reference (bedrock in the bottom or walls), but rarely in water column. The time spent by each fish in each behavioral category was summed.

Statistical analysis

The time of each behavioral category were organized in tables and individual estimated frequencies graphs. To verify variability in categorical data we made a box-plot showing medians and standard deviations of frequencies. In order to analyze the similarity between behavioral patterns, that is, whether the proportions (time spent in each behavioral category) are the same in different species, a one-way ANOVA was performed, followed by post-hoc Tukey's pairwise comparisons. We used frequency means for calculations and reliability index of 0.05 (Zar 1996). Statistical analysis was performed using PAST software (Hammer et al. 2001) and box-plot graph with Statistica software (version 12.0).

Results

Field observations

Table 1 presents the compared environmental and behavioral characteristics showed by copionodontine species.

The habitats of the troglolitic species are similar considering the physical characteristics. Water was generally dark/reddish, due to high levels of organic matter (Fig. 2a, b). Both solitary individuals and groups of fish, even in distinct caves, presented the same behavioral pattern: calm and exploratory swimming at the bottom (Fig. 2a), sometimes outside of the dens (exposed), or hidden between pebbles.

Regarding the epigeal species, *Copionodon pecten*, we also observed similar physical characteristics in Coisa Boa and Xavier rivers. Both solitary and gregarious fish exhibited basically the same behavioral pattern: calm and exploratory swimming, mainly at the bottom, exposed or under a shelter formed by bedrock (see the habitat at Fig. 2c).

Table 1. Description of habitat and space use by Copionodontinae. General description of habitat and space use of Copionodontinae catfishes from Chapada Diamantina, eastern Brazil. O.M., organic matter.

	Troglobites (<i>Glaphyropoma spinosum</i> and <i>Copionodon</i> sp. n.)	Epigean (<i>Copionodon pecten</i>)
Habitat	Pools with 0.2 to 0.5 m of depth. Water current from weak to moderate. Fig. 2a, b.	Pools or river stretch with 0.2 to 0.7 m of depth. Water current from weak to moderate. Fig. 2c.
Bottom river	Bedrock, sand, gravels, pebbles and boulders. Few amount of O.M.	Mainly bedrock and sand. Few gravels, pebbles and boulders. O. M. variable.
Activity	Stationary or exploratory swimming, sometimes counter-current.	Preference for exploratory swimming, sometimes counter-current or stationary.
Spatial distribution	Exposed in the bottom or hidden under pebbles, seldom in midwater, preference for shallow waters.	Exposed in the bottom or hidden under the shelf formed by bedrock, seldom in midwater, preference for shallow waters.
Gregarious/solitary habits	Solitary or in small groups.	Groups from three to 70 individuals, seldom solitary.

Spontaneous behavior

Statistical analysis shows that the space use behavior between copionodontine species is significantly different ($F=16.78$, $p<0.0005$), result corroborated by post hoc tests Tukey test (Table 2).

Time frequencies for each behavioral category, resultant from the spontaneous behavior, were organized in graphs of frequencies (Fig. 6). We produced a box-plot graph to explore the intraspecific/individual variation in these time frequencies (Fig. 7). The troglobitic *Glaphyropoma spinosum* exhibited the lowest variability (Fig. 6a, 7) in almost all categories, with exception to the hidden category, which was extremely wide and variable (Fig. 7). *Copionodon* sp. n. and the epigean *Copionodon pecten*, however, had higher intraspecific/individual variation (Fig. 6b, c) and wider data distribution (Fig. 7), with few outliers. All individual timings were summed up and are shown in Table 2.

Glaphyropoma spinosum is the most cryptobiotic species, having an evident preference for the hidden behavior category (76.9%, Table 2 and Fig. 6a). Only a single specimen did not show such behavior, remaining stationary almost all the time (specimen 11 - 98.5%). This species did not display midwater swimming.

In a general way, as observed for *Glaphyropoma spinosum*, the new *Copionodon* species also showed a hiding tendency (Table 2 and Fig. 6b – 44.4%). Nevertheless, it also remained for a great part of the time swimming in contact with the substrate (bottom + wall = 49.6%). If calculated together, bottom and wall swimming exceeds the time spent hiding, indicating a preference for swimming with the ventral region touching the substrate, a typical benthonic behavior.

The epigean *Copionodon pecten* also spent much time swimming in contact with the substrate (bottom + wall = 39.4% – Table 2) and hidden (32.6%). However, just like *Copionodon* sp. n., it shows a slightly preference for swimming with the ventral region touching the substrate. This species did not display midwater swimming.

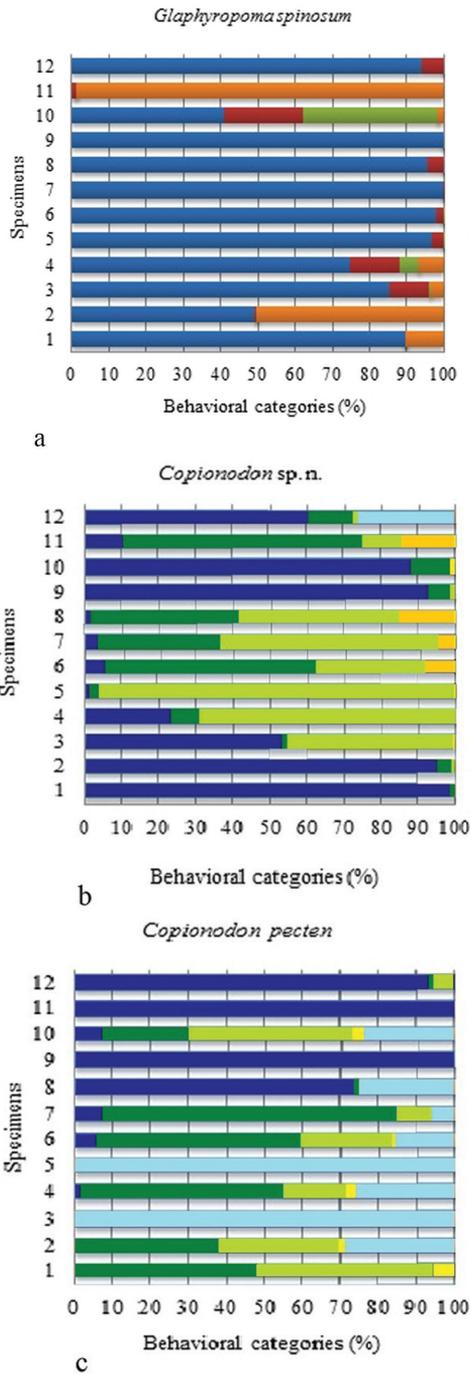


Figure 6. Individual frequencies of the behavioral categories recorded for copionodontine catfishes from Chapada Diamantina. Dark blue, hidden; light blue, stationary; dark green, bottom swim; light green, wall swim; dark yellow, midwater swim; light yellow, surface swim **a** *Glaphyropoma spinosum* **b** *Copionodon* sp. n. **c** *Copionodon pecten*.

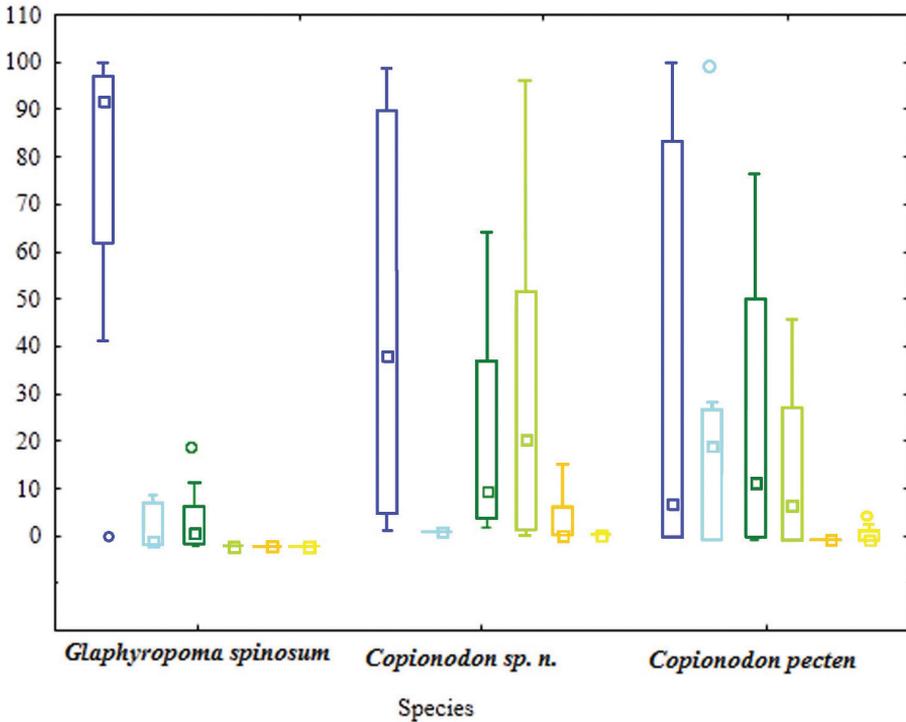


Figure 7. Box-plot (medians and standard deviations on Y axis) showing the spontaneous behavioral categories variability for Copionodontinae catfishes from Chapada Diamantina. o, outliers; dark blue, hidden; light blue, stationary; dark green, bottom swim; light green, wall swim; dark yellow, midwater swim; light yellow, surface swim.

Table 2. Frequencies of behavioral categories. Frequencies (%) of behavioral categories for Copionodontinae catfishes from Chapada Diamantina, eastern Brazil.

	Hidden	Bottom swim	Wall swim	Midwater swim	Surface swim	Stationary
<i>Glaphyropoma spinosum</i>	76.9	5.3	3.4	0	0	14.4
<i>Copionodon sp. n.</i>	44.4	20.1	29.5	3.5	0.2	2.3
<i>Copionodon pecten</i>	32.4	24.7	14.7	0	1	27.2

Discussion

Most studied troglotic fish with bottom-dwelling ancestors, show a trend in increasing midwater exploratory behavior in parallel with decreasing cryptobiotic behavior, that are considered behavioral specializations to cave life (Trajano 1993, Parzefall and Trajano 2010). However, both cave copionodontines herein studied presented cryptobiotic and benthonic behavior. The same pattern (strong cryptobiotic and benthonic) was described for other heptapterid cave catfishes: *Pimelodella spelaea* Trajano, Reis and Bichuette 2004 and a species of *Rhamdia* from northeastern Brazil (Bichuette and Trajano 2005). Considering the cryptobiotic behavior, three populations of the

armored cave catfish *Ancistrus cryptophthalmus* show burrowing behavior (Bessa and Trajano 2001), resembling that observed for subterranean species of Copionodontinae studied herein.

The strong cryptobiosis presented by the subterranean species *Glaphyropoma spinosum* is corroborated by field and laboratory observations, where only rarely the specimens were observed exposed, stationary on the aquarium bottom, and seldom exploring walls and water column. Despite the fact that hiding is a well-known strategy against visually oriented predators, it is not an effective tactic in environments with absence of light, such as caves. Moreover, in a high intraspecific competition situation hiding behavior may be disadvantageous, since it increases the time of search of food (Trajano 1989). However, *G. spinosum* cryptobiotic behavior may be related to the strong territorialism and photophobia. In fact, dens are apparently highly valuable resources, since it consists of one of the determinant factors for establishing dominance in *G. spinosum*, maybe allied to spawning periods (hypothesis to be tested), which always displays patrolling behavior as observed in laboratory.

For *Copionodon* sp. n., despite the great intraspecific variation, some behavioral patterns were detected, also exhibiting cryptobiotic and benthonic behavior. The same was described for troglobitic trichomictid *Ituglanis passensis* Fernández and Bichuette 2002, *I. bambui* Bichuette and Trajano 2004 and *I. ramiroi* Bichuette and Trajano 2004 (Bichuette 2003). Despite the weak surface or midwater exploratory behavior, *Copionodon* sp. n. displays an extended space use when compared to *G. spinosum*, since it expands its exploratory behavior to the aquaria walls. Phototactic and agonistic tests corroborate this trend: *Copionodon* sp. n. is less photophobic than *G. spinosum*, and less territorial as well.

With respect to the variability in the spontaneous behavior, it is clear that *Glaphyropoma spinosum* shows less intraspecific variation than *Copionodon* sp. n., also observed in their morphology: *G. spinosum* is more homogenous regarding melanic pigmentation and eyes size than *Copionodon* sp. n. The same trend was observed in phototactic behavior tests, in which *G. spinosum* exhibited very small individual differences (Rantin and Bichuette 2013).

The strong benthonic behavior displayed by the epigeal species, *Copionodon pecten* could be related to its foraging behavior and diet. Zanata and Primitivo (2013) observed a shift in *Copionodon pecten* diet and feeding behavior according to its ontogenetic stage: juveniles swim more on water column, feeding on drifting algae and other items; adults, on the other hand, are mainly benthonic, scratching algae and other items from substrate. Our laboratory observations were made only with adult specimens and we observed the same pattern. However, unlike proposed by Zanata and Primitivo (2013) we also observed many juveniles of *C. pecten* foraging in the substrate in field, with strong benthonic behavior.

Moreover, in a general way, it seems that the walls/substrate play an important role on the copionodontines' exploratory behavior: they probably use positive thigmotaxis, that is, the tendency to remain close to surfaces, to better choose where/how to explore the environment. Whenever exploring away from the den, all species preferred

to swim on aquarium walls, instead of extending it to midwater (“open field”), that is, they probably use thigmotaxis to choose the most secure areas of the test aquarium to explore. The highly photophobic spring cave fish *Chologaster papilliferus* Forbes, 1882 also uses thigmotactic responses to find secure dens, and usually seeks several levels of depth in order to carry out the touch responses (Weise 1957).

Therefore, *G. spinosum* and *Copionodon* sp. n. are cryptobiotic and benthonic, unlike most cave fish. Whether this pattern was retained from ancestors or consist of new acquisitions, such behaviors are probably important to help these species to cope with cave pressures and to successfully colonize the subterranean environment: both species form great groupings, with extremely high population densities (Bichuette 2012).

It is noteworthy that, unlike the great majority of troglobitic fish, the subterranean copionodontines, *Glaphyropoma spinosum* and *Copionodon* sp. n., alternate solitary/gregarious habits. The sympatric epigean *Copionodon pecten* is rarely observed alone, usually only when trapped in isolated pools (Zanata and Primitivo 2013). The spring cave fish *Chologaster papilliferus* display gregarious behavior, explained by a thigmotactic response: the specimens use this positive sthigmotactic behavior in order to recognize other individuals (sexual dimorphism in head and pelvis), and apparently, this is dependent on their sensory papillae distributed on body and mainly on head (Weise 1957). However, so far, most Brazilian cave catfishes are believed to be solitary, a plesiomorphic condition to some species (such as *Rhamdiopsis krugi* – Trajano and Bockmann 1999) and an autapomorphy for others, like *P. kronei* (Trajano 1989). Our results show that the comparisons must be more specific always considering the phylogenetic context.

Considering a more inclusive comparative context (Copionodontinae clade), cave copionodontine spontaneous behavior cannot be interpreted as specialization to isolation in the cave environment, since they do not present extended spatial exploratory behavior allied to reduction of cryptobiotic behavior, as observed for many cave fishes (Parzefall and Trajano 2010). Moreover, it is possible that after isolation in the subterranean environment, *Glaphyropoma spinosum* and *Copionodon* sp. n. retained the benthonic behavior and their spontaneous behavior can be interpreted as a plesiomorphic character-state in relation to Trichomycteridae clade, in according to the observed for the morphological traits, observed by Pinna (1998).

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Cave invertebrates in Espírito Santo state, Brazil: a primary analysis of endemism, threats and conservation priorities

Marconi Souza Silva¹, Rodrigo Lopes Ferreira²

¹ *Universidade Federal de Lavras, Departamento de Biologia, Setor de Zoologia Geral, Centro de Estudos em Biologia Subterrânea*

Corresponding author: *Marconi Souza Silva* (marconisilva@dbi.ufla.br)

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Abstract

The cave-dwelling invertebrates were studied according to their composition, biodiversity, distribution and threats in the Atlantic Forest Central Biodiversity Corridor, a priority area for conservation actions in Brazil. Twelve obligate cave species were found, plus 495 troglophile species. Araneae (103 spp.), Coleoptera (61 spp.), Diptera (56 spp.) and Lepidoptera (38 spp.) were the richest taxa. The richness was higher in the carbonate caves (63 spp., $sd = 16.7$) and the highest diversity in granitic caves ($H' = 2.68$, $sd = 0.5$). The spatial turnover was 63.45 and similarity less than 30%. The total richness was correlated with the linear extension of the caves ($R_s = 0.757$, $p \leq 0.05$). Surrounding area deforestation and religious and tourist use were the main threats. Emergency attention is recommended regarding protective actions, management and conservation of caves of extremely high biological importance.

Keywords

Atlantic forest, biodiversity, cave protection, subterranean habitats, troglobiont species

Introduction

In the state of Espírito Santo, Brazil, the degradation of the Atlantic Forest has been as widespread and intense as in the rest of the country. From the original native forest cover, there is currently only approximately 11% remaining (Ribeiro et al. 2009, SOS Mata Atlântica and INPE 2014). Despite this fact, the state is still an important area of the Brazilian Atlantic Forest, in relation to the conservation of animal and plant biodiversity. Currently, there are regional planning geographical units in this state which comprise a mosaic of land uses and key conservation areas, established in workshops organized by Conservation International. Furthermore, Espírito Santo contains an area of the Brazilian Atlantic Forest Central Biodiversity Corridor (Aguiar et al. 2005, Galindo and Câmara 2005).

The corridor is extremely diverse, sheltering many animal and plant species with restricted distributions, including threatened species (Brown 1972, Thomaz and Monteiro 1997, Thomas et al. 1998, Passamani et al. 2000). As an example, the tableland forests of the state (Reserva Biológica de Sooretama and Reserva Florestal de Linhares) still harbor a diverse, but poorly known fauna (Peixoto and Gentry 1990, Chiarello 1995). Thus, there are still many knowledge gaps regarding the geography of the biodiversity in the state, especially due to the lack of published information for many groups. As an example, there are the natural subterranean cavities (caves) that, up to now, are represented in very few publications concerning inventories of their species (Ruschi 1952, Souza-Silva et al. 2011a, Souza and Ferreira 2011).

The first study regarding caves animals in the Atlantic Forest was made in 1907 in Iporanga municipality (São Paulo state), with the description of *Pimelodella kronei* (Ribeiro 1907), a troglobiont catfish species. However, descriptions of new invertebrate cave fauna species in this biome were only continued in the mid-1930's (Costa-Lima and Costa-Leite 1953); until the end of the 1970's the studies were still of a taxonomic nature and restricted to a few caves in the south of São Paulo state.

It was only in the 1980's that more general studies started to be conducted, when Dessen et al. (1980) published surveys of invertebrate cave fauna, some of them in the Brazilian Atlantic Forest. Subsequent studies were made in Ubajara municipality (Ceará state), southern Minas Gerais state, southern Bahia state (limestone province of Rio Pardo) and the Vale do Ribeira limestone province in the extreme south of São Paulo and northeast Paraná regions (Souza-Silva et al. 2015). Only after 2010 were geographically broader studies regarding the invertebrate cave fauna in the Brazilian Atlantic Forest conducted (CEBS 2015).

Caves are subterranean environments which harbor a rich fauna living with low food resource availability, constant temperatures and high humidity (Culver and Pipan 2009, Souza-Silva et al. 2011b, Schneider et al. 2011). Many troglobiont cave species have restricted distributions and limited dispersal (Culver and Pipan 2009); consequently, they can be strongly affected by even moderate (human) changes in their environment. Furthermore, due to the high environmental stability of subterranean caves, such environments become highly susceptible to impacts, since this stable condition can often be easily unbalanced. Therefore, any direct or indirect impacts on

caves can cause serious damage to the fauna and the physical integrity of these environments (Souza-Silva et al. 2015).

The characteristics most often mentioned to explain why some species are more vulnerable to extinction than others include small population sizes, low geographic distribution, specialized habitat requirements (Pimm et al. 1988, Purvis et al. 2000, Webb et al. 2002), large body sizes (McKinney 1997) and slow life histories (small broods, slow growth rates, late maturity, long inter-birth intervals, high survival rates) (MacArthur and Wilson 1967). Species may also become rare, and consequently vulnerable to extinction, because they are often hunted and may have their habitats destroyed and/or polluted (Whitmore and Sayer 1992, Bodmer et al. 1997, Isaac and Cowlshaw 2004).

Many troglobiont species have low population growth rates and in this case, strong environmental changes may increase the risk of extinction (Culver and Pipan 2009). The main threats come from the destruction of their habitats by mineral exploitation, tourism, vandalism, heavy metals, herbicide or pesticide pollution, eutrophication and biological invasions (Tercafs 1992, Ferreira and Horta 2001, Parise and Gunn 2007, Souza-Silva et al. 2015). Thus, many species that inhabit caves can be considered threatened and/or vulnerable to extinction due to habitat specificity that operates by selecting different ecological-evolutionary specializations (trogloxene, troglophile and troglobiont) to the hypogean conditions and environmental resources (Sket 2008).

The present study sought to evaluate the composition, richness, diversity, turnover and similarity of invertebrate cave fauna in the state of Espírito Santo, Brazil, an important geographical unit for conservation in the Atlantic Forest. Thus, we attempted to also evaluate the human changes to these caves, using a Cave Conservation Priority index (Souza-Silva et al. 2015), in order to suggest general conservation and management actions for the caves and their surrounding forest cover.

Materials and methods

Study area

The caves investigated in this study are located in the Atlantic Forest Central Biodiversity Corridor in Espírito Santo, Brazil, a priority area for conservation (Galindo and Câmara 2005). The rock types in which the sampled caves were developed were granite (11 caves), gneiss (1 cave), limestone (1 cave) and marble (2 caves) (Fig. 1 and Table 1).

Sample data

Only one visit to each cave was conducted and invertebrates present in the caves were collected manually in all existing potential biotopes and plotted on a schematic sketch of each cave (Souza-Silva et al. 2011a). Samples were collected with the aid of tweezers, brushes and entomological nets, prioritizing microhabitats such as driftwood, guano deposits, under rocks and damp locations. All organisms found were identified to the

Table I. Location (UTM) and characteristics of 15 caves in the state of Espírito Santo, Brazil.

Cave	Municipality	X	Y	L	A (m)	H	SE (m)	SV
A. Pansini	Vargem Alta	285168.01	7711062.66	M	450	PS	300	M
Casa Branca	Itaimbe-Itaguaçu	305381.99	7830778.80	Gr	160	DC	15	P
Didi Vieira	Afonso Cláudio	284809.28	7766144.11	Gn	662	DC	79	SF
Evald	Domingos Martins	320007.63	7747606.91	Gr	705	DC	23	M
Faz. do Dr Saulo	Ecoporanga	302473.87	7969940.28	Gr	223	PS	60	P
Faz. Paraíso	Ecoporanga	306636.61	7957526.01	Gr	338	DC	13	SF
Henrique Altoé	Jaciguá	289929.20	7709392.59	Gr	600	PS	90	P
João Buteco	Ecoporanga	308438.31	7974280.89	Gr	250	DC	25	P
Limoeiro	Conceição de Castelo	273406.68	7733590.71	C	502.8	DC	600	P
Michele	Pancas	311940.58	7872453.45	Gr	97	PS	60	P
Mirante	Vargem Alta	285168.01	7711062.66	M	650	PS	30	SF
Represa	Santa Teresa	322639.34	7808340.40	Gr	626	PS	25	P
Rio Itaúnas	Pedro Canário	395452.20	7977430.06	Gr	301	PS	41	P
Ruschi	Santa Tereza	339370.99	7791692.36	Gr	672	TS	30	SF
Santa Bárbara	Venda N. Imigrantes	275936.82	7747596.29	Gr	1116	WT	80	M

(L) lithology, (A) altitude, (H) hydrology, (SE) sampled linear extension, (SV) 250m surrounding vegetation, (DC) dry cave, (TS) temporary stream, (WT) water table, (PS) perennial stream (Gr) granite (Gn) gneiss, (M) marble and (C) calcareous, (SF) secondary forest, (P) pasture and (M) monoculture.



Figure 1. Distribution of 15 caves in the Atlantic Forest in the state of Espírito Santo, with invertebrate fauna inventoried in this study. Source: SOS Mata Atlântica (2011).

accessible taxonomic level and grouped into morphotypes (Souza-Silva et al 2011a). The trophic characterization of caves was conducted concurrently with the invertebrate collections. For such, all organic resources present were noted and when possible their pathways to cavities characterized. The quality or quantity of food was not calculated and analyzes of water and soil nutrients were also not carried out.

Uses and environmental changes (impacts) in the caves and surroundings were evaluated based on forms filled out during visits (Simões et al. 2014, Souza-Silva et al. 2015).

Cave invertebrate community structure

To standardize the richness values used in the comparisons of caves, they were relativized according to the linear development and horizontal extension of the entrances of each cave (biological variable/cave linear development/ Σ entry width) (Souza Silva et al. 2011a). The relative richness categorizes the number of species as a function of the cave length and the extension of the entrances. Thus, this variable seeks to reduce excessive contribution of the para-epigeal community by considering the entrance extension in this analysis. It is expected that in the caves with extended entrances there is a great contribution by the para-epigeal communities due to increased contact with the external environment (Prous et al. 2004, Prous et al. 2015).

Statistical analysis

The alpha diversity values (α) of invertebrate communities associated with each cave were calculated using the Shannon-Weaver index (Magurran 2004). The beta diversity (β or turnover) was calculated, using the presence and absence of data, by the Harrison ratio (1992). $\beta_{\text{Harrison}} = \{[(S/\alpha)-1]/(N-1)\} * 100$, where S = total number of species, α = average richness and N = number of samples. This measure ranges from 0 (no turnover) to 100 (each sample has a single set of species) (Magurran 2004). The Berger-Parker dominance index was used for assessing the relative importance of dominant species in the communities (Magurran 2004). The similarities among the cave fauna were obtained through the Bray-Curtys index (Magurran 2004). The Spearman correlation (R_s) was used to detect possible relationships between total species richness and cave horizontal projection and entrance extension variables. Possible differences between richness and relative and total diversity among caves in carbonate (limestone and marble) and granitic rock (granite and gneiss) were evaluated using the Kruskal-Wallis test (Zar 1984). The software used for the analysis was PAST (Hammer et al. 2001).

Cave Conservation Priorities

To assess the status of threats to biodiversity in caves of the Atlantic Forest in Espírito Santo, we used the Cave Conservation Priority index (CCP i) proposed by Souza-Silva

et al. (2015). The *CCPi* provides a vulnerability score for each cave using the overlap of biological relevance (BR) and human impact degree (HID) (Souza-Silva et al. 2015). The biological relevance of the caves can be determined through the superimposition of three variables: troglobiont species richness ($T_{gb}R$), total and relative troglophile richness ($T_{tr}R$ and $R_{tr}R$). For each of these variables categories as weights were used, extremely high (weight 4), high (weight 3), average (weight 2) and low (weight 1) to facilitate valuations. Based on the highest sum value for these weights (8) ($BR_{weight} + HID_{weight}$) four cave vulnerability or conservation priority categories were created. Such categories include extremely high (weight ≥ 6), high (4-5.99) average (2 – 4.99), and low (≤ 1.99) (Souza-Silva et al. 2015).

Human impacts

The human modifications surveyed in this study were classified in relation to *uses* and *impacts*. Tourist and religious activities were considered uses, impacts being trampling, illumination and construction resulting from these activities (Souza-Silva et al. 2015).

Environmental changes (impacts) were defined for each cave in function of the presence or absence of modifications inside and in the surroundings. Surroundings was considered as a 250 meter radius projected from the cave's external contour, according to Brazilian law (CONAMA n° 347, September, 10th, 2004, Souza-Silva et al. 2015). The impacts were considered as those modifications that could potentially lead to *depletion* (a), *enrichment* (b) or *alteration* (c) in the microhabitats, organic resources and/or cave fauna (Souza-Silva et al. 2015).

The urgent conservation and management actions for the caves and their surrounding forest cover were based on six criteria: (1) microbiological research (suggested for caves with probable occurrence of human pathogenic fungi), (2) defining the abundance of troglobiont species, (3) recovery of the surroundings (suggested for caves with deforested surroundings area), (4) management plan (suggested for caves with intense human use), (5) maintenance of the surroundings using Private Natural Heritage Reserves-PNHRs, (suggested for caves with preserved surroundings area or in advanced succession), (6) compensatory measures (suggested for caves made completely uncharacteristic as a result of human activities).

Private Natural Heritage Reserves (PNHRs) is a category in the current National Conservation Unit System (Law 9.985 of 2000 – SNUC) that supports the creation of protected areas of variable sizes and uses (Maciel 2000).

Karstic areas for conservation priorities

The results of the *CCPi* associated with troglomorphic traits and troglobiont species richness and distribution were used to indicate karst areas that deserve attention with respect to conservation needs.

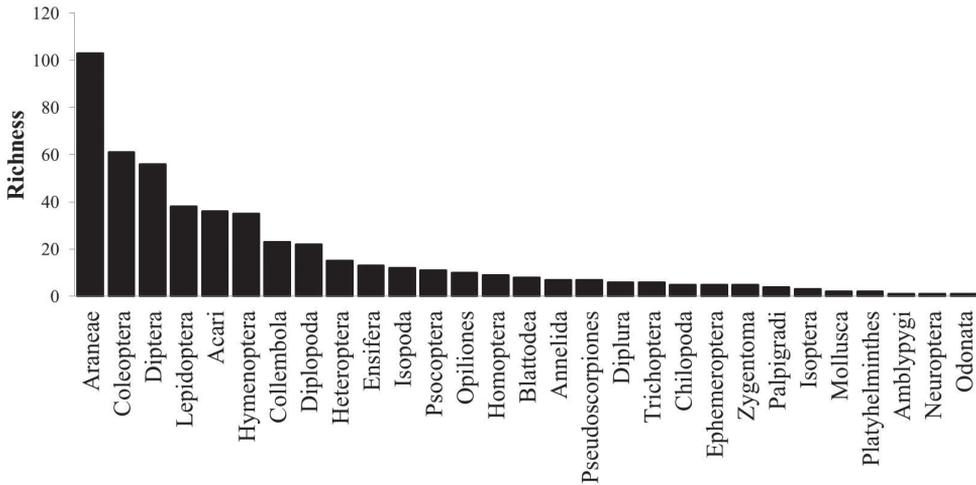


Figure 2. Composition and richness of invertebrate taxa collected in 15 caves in the state of Espírito Santo, Brazil.

Results

Invertebrate diversity and distribution

507 invertebrate species were collected, distributed in at least 121 families (Table 2). The taxa Acari (36 spp.), Araneae (103 spp.), Opiliones (10 spp.), Diplopoda (22 spp.), Collembola (23 spp.), Coleoptera (61 spp.), Diptera (56 spp.), Lepidoptera (38 spp.), Hymenoptera (35 spp.), Heteroptera (15 spp.), Ensifera (13 spp.), Isopoda (12 spp.) and Psocoptera (11 spp.) were the richest (Fig. 2).

Twelve species with troglomorphic traits were found, all arthropods, with distributions restricted to six caves: one isopod (Trachelipodidae) and one spider (Ochiroceratidae) both from a granitic cave, in Santa Teresa municipality; a silverfish (Zygentoma: Lepismatidae) and an isopod (Isopoda: Plathyarthridae: *Trichorhina* sp.) both from a granitic cave in Ecoporanga municipality; another silverfish species (Zygentoma: Lepismatidae) from a granitic cave in Pedro Canário municipality; a palpigrade (Eukoeneriidae: *Eukoeneria spelunca*, Souza and Ferreira 2011), a harvestman (Opiliones: Escadabiidae) and a millipede (Polydesmida: Trichopolydesmidae) from a granitic cave in Jaciguá municipality and two millipedes (Spirostreptida: Pseudonannolenidae: *Pseudonannolene* sp) and a Polydesmida: Cryptodesmidae from a marble cave in Vargem Alta municipality and another harvestman (Opiliones: Escadabiidae) from a carbonate cave in Conceição do Castelo municipality (Table 3). Most of the troglomorphic specimens collected were sent to specialists and are under description (Fig. 3).

The total richness varied between 17 to 79 species, and the average species richness was 51 species. The relative richness varied between 0.01 to 1.03, and the average relative species richness was 0.25 (Figures 4 and 5). The total abundance varied from 50 to 9,029 individuals per cave and the average abundance was 1,421 individuals. The dominance varied from 0.06 to 2 and the average dominance was 0.13. The diversity

Taxa	Family/subfamily	DV	FP	Mi	L	RE	JB	CB	Ru	E	SB	FS	Mi	AP	HA	RI	
	Theridiidae	+			+	+	+	+	+	+	+	+			+	+	
	Theridiosomatidae		+	+								+	+	+	+		
	Uloboridae							+	+		+						
Opiliones	Gonyleptidae		+		+				+		+		+	+	+		
	Escadabiidae														+		
Palpigradi	Eukeneniidae	+		+					+					+			
Pseudoscorpiones	Chernetidae	+	+	+					+		+			+	+	+	
Diplopoda	unidentified															+	
	Cryptodesmidae	+	+	+											+		
	Pseudonannolenidae			+	+						+			+	+		
	Pyrgodesmidae								+		+			+		+	
	Rinocricidae				+												
Polydesmida	unidentified					+					+					+	
	Trichopolydesmidae															+	
Spirobolida	unidentified				+			+								+	
Geophilomorpha	unidentified	+				+											
Litobiomorpha	unidentified								+								
Polyxenida	unidentified				+												
Symphyla	unidentified	+	+	+		+			+		+	+		+			
Archaeognatha	Meinertellidae			+													
Blattodea	unidentified			+	+		+		+		+	+	+	+		+	
Coleoptera	unidentified	+	+	+	+		+		+		+		+			+	
	Carabidae	+		+					+		+		+				
	Chrysomelidae				+												
	Dermestidae	+															
	Dryopidae			+													
	Elateridae				+												
	Elmidae														+		
	Histeridae			+			+								+	+	+
	Lampyridae								+		+						
	Leiodidae										+						
	Cholevidae										+						
	Pselaphidae	+	+	+					+		+				+	+	
	Scarabeidae								+								
Staphylinidae		+	+	+	+			+	+	+	+	+		+	+		
Tenebrionidae	+	+	+	+		+			+		+			+		+	
Collembola	unidentified	+	+	+	+	+			+		+	+	+	+	+	+	
Diplura	Anajapygidae								+								
	Camptodeidae	+	+						+								
	Japygidae								+								
	Projapygidae	+	+														
Diptera	unidentified	+		+	+		+				+		+	+		+	
	Agromyzidae			+	+				+	+	+			+			
	Calliphoridae								+					+			
	Cecidomyiidae							+									
	Chironomiidae													+			

Taxa	Family/subfamily	DV	FP	Mi	L	RE	JB	CB	Ru	E	SB	FS	Mi	AP	HA	RI
	Culicidae	+						+			+					+
	Dixidae				+						+		+	+		
	Dolychopodidae	+			+								+			
	Drosophilidae						+	+				+	+			+
	Keroplastidae		+										+			+
	Lauxanidae				+						+					+
	Lonchaeidae						+									
	Milichidae															+
	Mycetophilidae														+	
	Nematocera	+			+	+			+		+	+	+			
	Phoridae		+			+			+	+	+	+	+	+	+	+
	Psychodidae		+	+	+		+		+			+	+		+	+
	Sciaridae	+		+	+	+						+				
	Simulidae			+									+	+		
	Sphaeroceridae				+											
	Empididae			+												
	Syrphidae	+				+										
	Tipulidae	+							+			+				
Ensifera	Phalangopsidae		+	+	+	+		+	+	+	+	+	+	+	+	+
	Tetigoniidae	+														+
Ephemeroptera	unidentified			+									+	+		+
	unidentified			+												+
	Cydnidae		+						+		+	+			+	+
Hemiptera/ Heteroptera	Gelastocoridae					+										
	Naucoridae			+												
	Emesiinae	+	+		+					+			+	+		+
	Reduviinae	+	+	+	+	+			+	+	+	+	+	+	+	+
	Vellidae										+	+		+		
Hemiptera/ Homoptera	unidentified			+												
	Cixidae	+	+	+		+			+							
	Membracidae	+									+					
	unidentified	+	+	+	+				+		+			+	+	
	Brachonidae										+					+
	Evaniidae				+											
Hymenoptera	Formicidae	+	+	+	+	+	+	+	+	+	+	+	+		+	+
	Halictidae	+														
	Mutillidae				+											
	Sphecidae	+										+				+
	Vespidae				+											
Isoptera	Termitidae	+		+		+							+	+	+	
	unidentified	+			+				+		+	+		+		
Lepidoptera	Geometridae			+												
	Hesperiidae	+										+				
	Noctuidae	+		+				+	+					+		

Taxa	Family/subfamily	DV	FP	Mi	L	RE	JB	CB	Ru	E	SB	FS	Mi	AP	HA	RI
	Nymphalidae	+														
	Tineidae	+	+	+	+	+		+	+	+		+	+	+	+	+
Neuroptera	Myrmeleontidae	+			+								+			
Odonata	unidentified			+												
Psocoptera	unidentified								+		+					
	Lepidopsocidae										+					
	Pseudocaeciliidae								+							
	Psyllipsocidae					+							+	+		
Trichoptera	unidentified					+							+			
	Hydropschidae											+	+			
Thysanura	Lepidotrichidae								+							
	Lepismatidae							+				+				+
Mollusca	unidentified			+												

Table 3. Biological characteristics and priority actions for 15 caves in the state of Espírito Santo, Brazil. Legend: (S) total richness (A) abundance, (D) dominance, (H) diversity, (E) equitability, (TR) organic resources (HG) hematophagous guano (PD) plant debris (CG) carnivore guano, (FG) frugivore guano (R) roots. (PA) priority actions (1) microbiological research, (2) define status of endemic species, (3) recovery of the surroundings, (4) management plan, (5) maintenance of the surroundings, (6) compensatory measure. (T) troglomorphic taxa.

Cave	S	A	D	H	E	TR	PA	T
Archimedes Pansini	66	760	0.08	2.99	0.71	HG	2, 3	<i>Eukoenia spelunca</i> , <i>Pseudonnanolene</i> sp. n., <i>Cryptodesmidae</i> sp. n., <i>Escadabiidae</i> sp. n., <i>Trichorbina</i> sp. n.
Casa Branca	41	183	0.2	2.36	0.64	?	6	
Didi Vieira	64	231	0.06	3.51	0.84	PD	5	
Evald	17	50	0.19	2.23	0.79	PD	3	
Fazenda do Dr. Saulo	46	1162	0.11	2.61	0.68	HG	2, 3	<i>Zygentoma</i> and <i>Trichorbina</i> sp. n.
Fazenda Paraiso*	40	797	0.18	2.12	0.57	CG	1	
Henrique Altoé	50	854	0.14	2.44	0.63	R, HG	3, 4	<i>Trichopolydesmidae</i> sp. n.
João Buteco	17	646	0.16	2.17	0.76	HG	3	
Limociro	78	4074	0.20	2.38	0.54	HG	2, 3, 4	Opiliones
Michele	73	245	0.06	3.48	0.81	R, HG	3	
Mirante	45	1920	0.2	2.11	0.55	HG	5	
Represa	43	297	0.1	2.86	0.76	R	3, 5	
Rio Itaúnas	49	9029	0.12	2.28	0.59	FG	2, 3	<i>Zygentoma</i> and <i>Trichorbina</i> sp. n.
Ruschi	79	462	0.09	3.13	0.72	PD; HG	2, 5	<i>Trachelipodidae</i> and <i>Araneae</i>
Santa Bárbara	61	617	0.08	2.95	0.72	HG	3, 4, 5	
Average	51.3	1421	0.13	2.64	0.60			
Standard Deviation	19	2330	0.05	0.48	0.1			

* Probable occurrence of strains of *Histoplasma* sp. associated with the guano. Two team members collecting fauna had contact with guano, presented symptoms and diagnosed positive for Histoplasmosis. Farm owner reported that a nephew who had visited the caves showed Histoplasmosis symptoms, but without a confirmed diagnosis.



Figure 3. Some of the troglomorphic invertebrates sampled in 15 caves in Atlantic forest at Espírito Santo state, Brazil. **A** Escadabiidae, **B** Trachelipodidae, **C** *Pseudonannolene* sp., **D** Cryptodesmidae, **E** Trichopolidesmydae, **F** Zygentoma, **G** *Trichorhina* sp.

varied between 2.11 to 3.51, and the average diversity was 2.64 (Table 3 and Fig. 4). The β diversity was 63.45. The similarity of the fauna was low, less than 30%. The total richness correlated positively and significantly ($R_s = 0.757$) with the linear extension of the caves (Fig. 6A). However, when the carbonate caves were excluded from the sample, there was no significance in the test (Fig. 6B).

The average richness was higher in the limestone caves (marble and calcareous) (63 spp., sd = 16.7) and the diversity was higher in the granite caves ($H' = 2.68$, sd = 0.5), but there were no significant differences in average richness and diversity between carbonate and granite caves (Fig. 7).

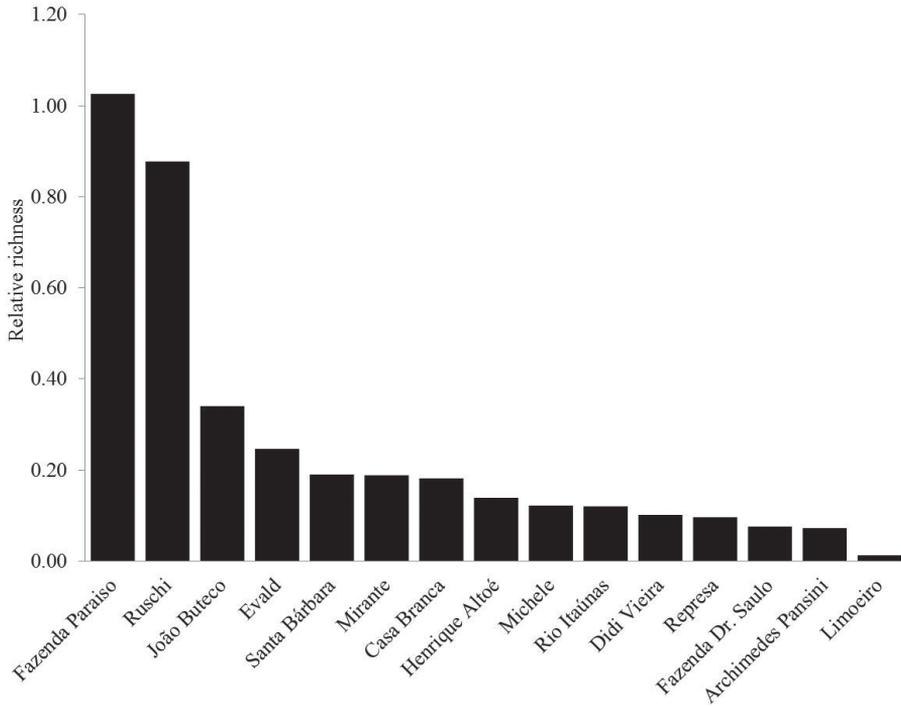


Figure 4. Relative richness of the invertebrate fauna in 15 caves in the state of Espírito Santo.

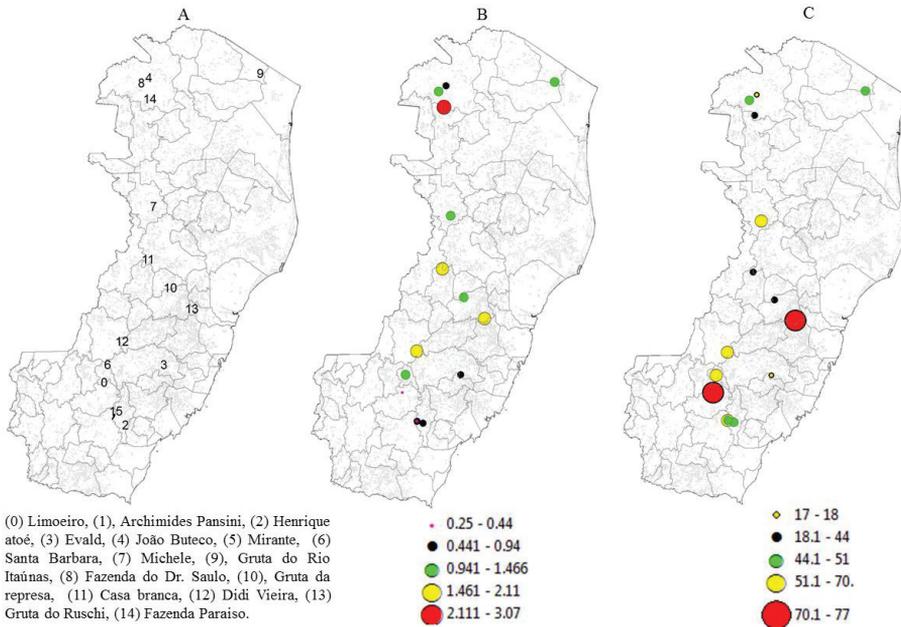


Figure 5. **A** Distribution of caves, **B** relative richness and **C** total richness of the 15 caves of the state of Espírito Santo, Brazil.

Table 4. Qualification of use occurrence * and human alteration weights in 15 caves in the state of Espírito Santo, Brazil.

Cave	1*	2*	3	4	5	6	7	8	9	10	Sum	IW
Archimedes Pansini		+	1		7		7			3	18	H
Casa Branca	+		1		7	4	7		7		26	EH
Didi Vieira											0	L
Evald			1								1	L
Fazenda do Dr. Saulo			1					6			7	A
Fazenda Paraíso											0	L
Henrique Atoé			1								1	L
João Buteco			1				7				8	L
Limoeiro	+	+	1	4	7	4	7		7	3	33	EH
Michele			1								1	L
Mirante											0	L
Represa			1								1	L
Rio Itaúnas			1			4	7	6			18	H
Ruschi											0	L
Santa Bárbara	+	+	1		7	4	7				19	H

Legend: Religious use (1), tourist use (2), surrounding deforestation up to 250m (3), detonations inside (4), trampling inside (5), construction inside (6), trash inside (7), subterranean drainage exploitation (8), electric lighting (9) depredation of speleothems (10). (IW) impact weight categories. Extremely high (EH), High (H), Average (A), Low (L).

Table 5. Categorization of the caves regarding biological relevance, impact degree and vulnerability of their communities. Caves with troglomorphic species (*).

Cave	Troglomorphic relevance	Biological relevance	Impacts degree	Vulnerability to protect
Didi Vieira	Low	High	Low	Average
Evald	Low	Low	Low	Average
Faz. Paraíso	Low	High	Low	Average
Henrique Atoé	Low	Average	Low	Average
João Buteco	Low	Average	Low	Average
Michele	Low	High	Low	Average
Mirante	Low	High	Low	Average
Represa	Low	Average	Low	Average
A. Pansini	Extremely High	Extremely high	High	Extremely high
Faz. do Dr. Saulo*	Average	Extremely high	Average	Extremely high
Limoeiro*	Low	Extremely high	Extremely high	Extremely high
Rio Itaúnas*	Average	Extremely high	High	Extremely high
Ruschi*	Average	Extremely high	Low	Extremely high
Casa Branca	Low	Average	Extremely high	High
Santa Bárbara	Low	Average	High	High
Categories	%	%	%	%
Extremely high (%)	6.7	33.3	13.3	33.3
High (%)	0	26.7	20	13.3
Average (%)	20	33.3	6.7	53.3
Low (%)	73.3	6.7	60	0

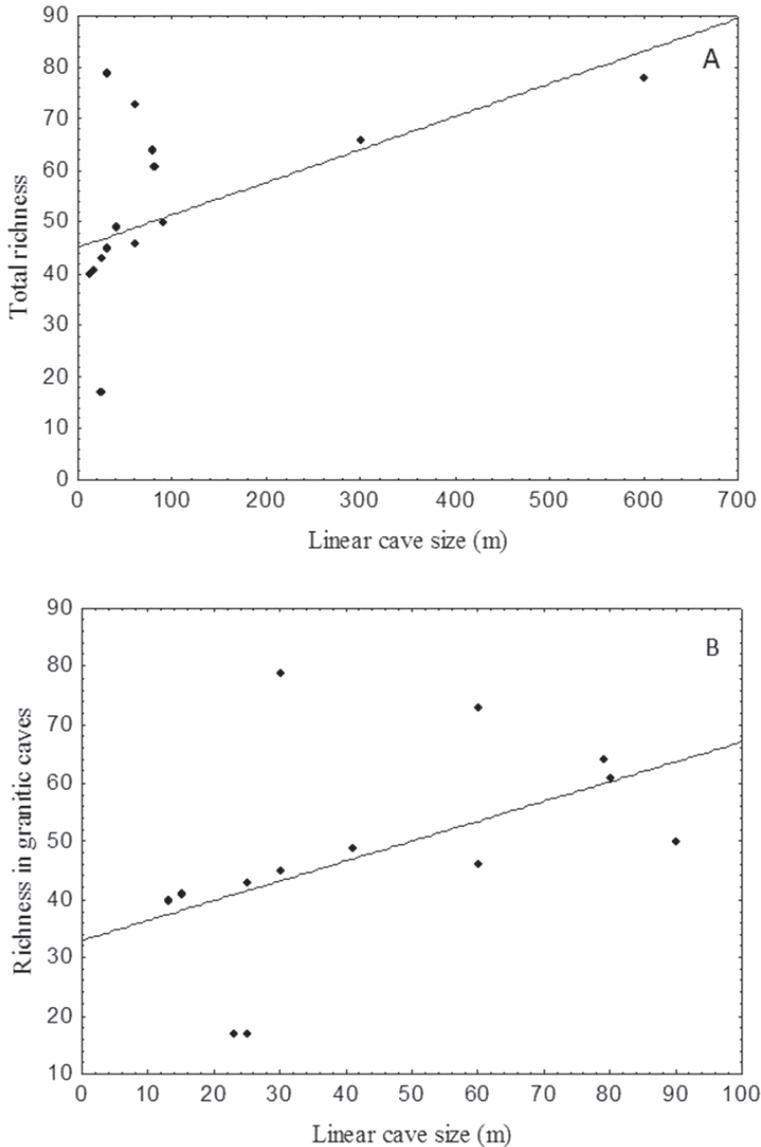


Figure 6. **A** Significant relationship of the increased richness of collected invertebrates with the increase in size of caves in limestone and granitic rocks and **B** no significant relationship without limestone caves in the state of Espírito Santo.

Trophic chain basis

Qualitatively, the food resources for invertebrates were variable regarding to the type and quantity present in different caves. The most frequent were the plant detritus accumulated near the entrances, bat guano (from hematophagous, insectivorous, frugivo-

Table 6. Criteria used in defining cave priorities for conservation actions based on 15 caves located at Espírito Santo.

Priority	Degree	Criteria
1	Extremely high	Expected relative occurrence of 3.76 or more troglotibiotic/trogломorphic species of wide or narrow distribution; total species richness more than 61 species; relative richness more than 0.76 (biological relevance greater than 6.1 points) and sum of impact weights more than 25 points.
2	High	Expected relative occurrence from 2.6 to 3.75 troglotibiotic/trogломorphic species of wide or narrow distribution, total richness between 41 to 50 species; relative richness between 0.6 and 0.75 species (biological relevance between 4.1 and 6 points) and the sum of impact weights between 16.6 and 24.9 points.
3	Average	Expected relative occurrence from 1.26 to 2.5 troglotibiotic/trogломorphic species of wide or narrow distribution; total richness between 21 and 40 species; relative richness between 0.26 and 0.5 species (biological relevance between 2.1 and 4 points) and the sum of impact weights between 8.26 and 16.5 points.
4	Low	Expected occurrence from 1.25 or less troglotibiotic/trogломorphic species; total richness less than or equal 20 species; relative richness less than or equal 0.25 species (biological relevance less than 2.1 points) and the sum of impact weights less than or equal 8.25 points.

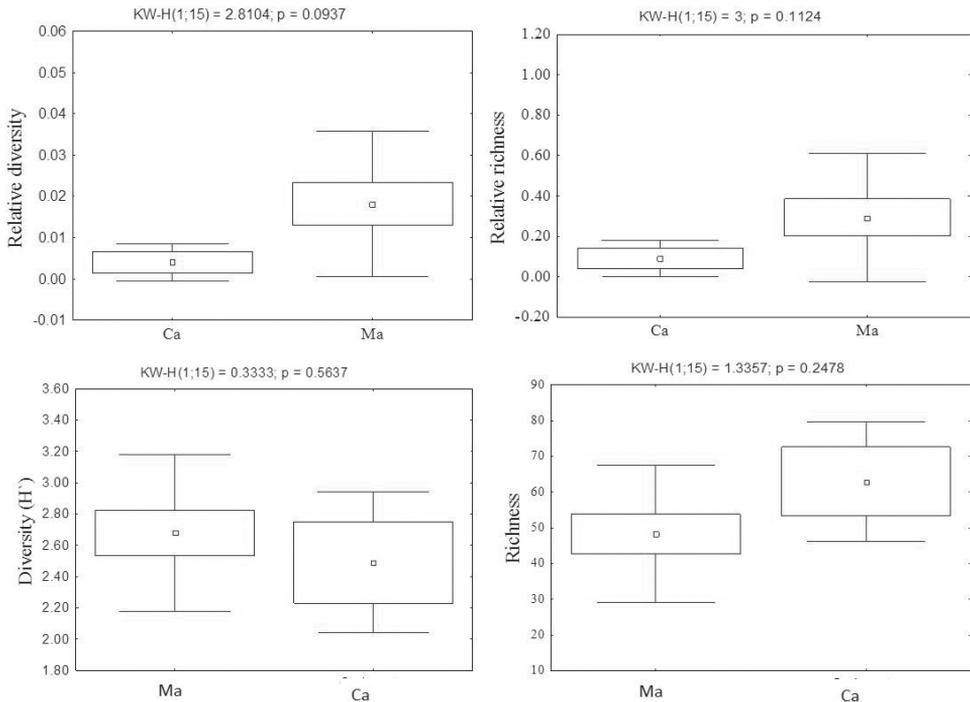


Figure 7. Almost significant differences between the diversity and total and relative richness of invertebrates in caves that develop in carbonate rocks and magma in the state of Espírito Santo. Average, \pm SE, \pm SD.

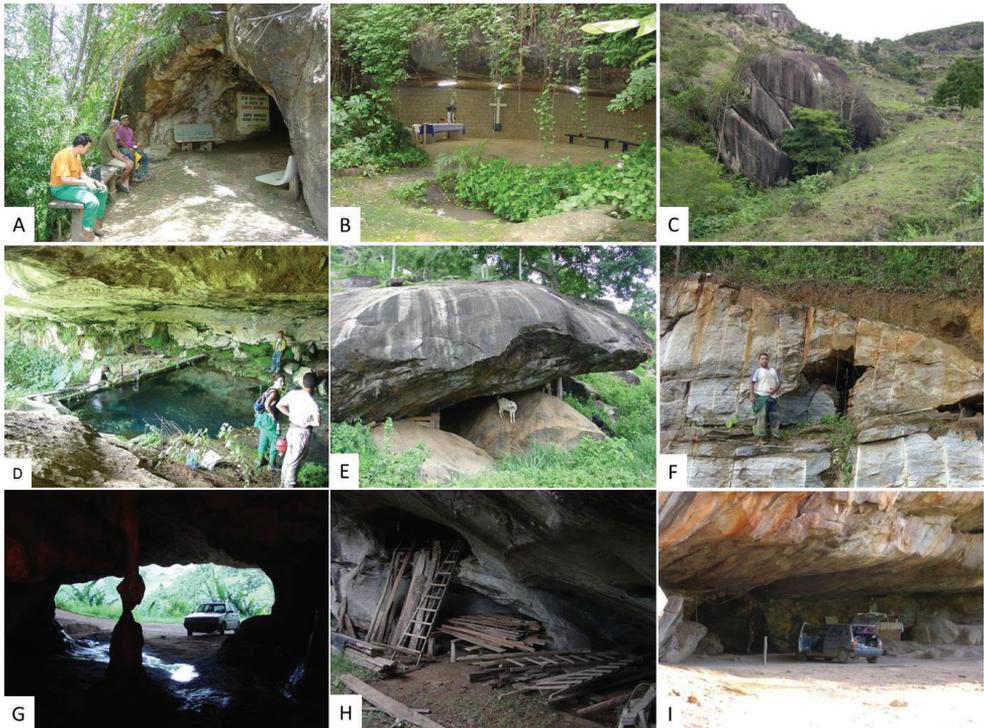


Figure 8. Human alterations in caves of Espírito Santo, Brazil. **A** religious use in granite cave in Venda Nova dos Imigrantes **B** transformation of granitic cave into a church in Itaimbé-Itaguassu **C** deforestation surrounding cave in Ecoporanga **D** drainage exploitation in granite cave near Pedro Canário **E** use of cave as goat corral **F** road construction destroying cave chambers in Vargem Alta **G** and **I** Limoeiro cave entrance with religious and tourist use in Conceição de Castelo **H** using limestone cave as a timber-yard in Vargem Alta.

rous and carnivorous bats) and roots of external vegetation accessing the cave galleries. The presence of visible food resources for invertebrate fauna was not detected only in the Casa Branca cave. This cave has been transformed into a small church with cemented floors and walls, electric lighting, an altar and wooden benches (Fig. 8B).

Biological relevance, human impact and vulnerability

Human changes (threats) observed were religious use, tourist use, deforestation of the surroundings, collapse by detonation, trampling, construction, garbage, subterranean drainage exploitation, electrical lighting and degradation of speleothems (Table 4, Fig. 8).

Regarding the biological relevance, 33% of the caves presented extremely high relevance, 26.7% high, 33% average and 6.7% low (Table 5, Fig. 9). As for the impact weights, 13.3% of the caves had an extremely high impact degree, 20% high impact

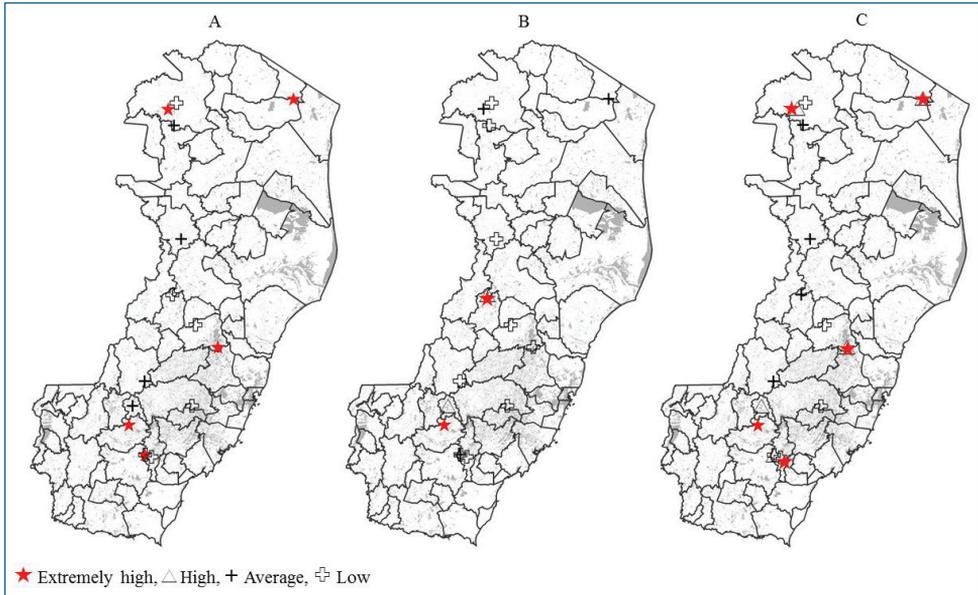


Figure 9. **A** Distribution of cave biological relevance **B** cave impacts category and **C** cave fauna vulnerability in the state of Espírito Santo. Gray shading on maps represents remnants of the Atlantic Forest.

degree, 6.7% average impact degree and 60% low impact degree. As to vulnerability, 33.3% showed extremely high vulnerability, 13.3% high vulnerability, 53.3% average vulnerability and no cave had a low vulnerability (Table 5, Fig. 9). Table 6 show the summary of the criteria used to assign each cave a conservation priority category.

Suggested conservation actions

Microbiological research in caves with a suspected presence of *Histoplasma* sp. was suggested only for the Fazenda Paraíso cave, since two members of the collecting team who had contact with guano presented symptoms and were diagnosed positive for histoplasmosis, including one of the authors (RL Ferreira). The farm owner also reported that a nephew who had visited the cave had a recurring fever (one of the histoplasmosis symptoms), but did not have a confirmed diagnosis.

Studies to define the population size and the real threat status of the troglobiont and endemic species were suggested for five caves. Recovery of cave surroundings that had sustained deforestation activity was suggested for nine caves, with the possibility of using permanent fruit culture (especially cocoa) mixed with native trees, because it can additionally function as an economic and ecological service. Management plans for caves with tourist and/or religious use was suggested for three caves. Maintenance of cave surroundings to preserve external vegetation was suggested for five caves (Private Natural Heritage Reserves-PNHRs). Environmental compensatory measures resulting from completely uncharacteristic cave habitat due to human activities were suggested for one cave (Table 3).

Suggested karst areas for conservation priorities

Three karst areas were identified as priorities for conservation action: granitic areas with caves located in the extreme north of Espírito Santo state, in the municipalities of Pedro Canário, and Ecoporanga (24K-395452/7977430), since the caves contain at least three troglomorphic species and face human modifications such as deforestation, agriculture, livestock and groundwater water exploitation. Another area is the granitic mountain rocks with caves in Santa Teresa municipality (24K-339370/7791692), since it presents at least two troglomorphic species and faces human impact, such as deforestation, agriculture, livestock, subterranean drainage alteration. The third area comprises the carbonate area with caves in Castelo, Vargem Alta and Cachoeiro do Itapemirim municipalities (24K-285168/7711062), with at least five species of troglobiont/troglomorphic species and faces human alterations, such as religious and tourism uses, deforestation, mining activities and road construction.

Discussion

Cave invertebrate community structure

The composition of the invertebrate cave fauna evaluated in this study, at least to the family level, is very similar to that registered for other caves in the Brazilian Atlantic Forest (Pinto da Rocha 1995, Trajano 2000, Souza-Silva and Ferreira 2009, Souza-Silva et al. 2011c, d). However, the cave fauna collection methodology employed in Brazil until the 1990's apparently has not resulted in an effective sampling of the fauna (due to the small number of species recorded) (Souza-Silva et al. 2011a and c). Furthermore, works carried out to the 1980's did not have any information regarding the abundance or diversity, and those related to fauna richness are underestimated.

It is important to consider that the work was developed almost two decades ago, in a scenario where the main goal was to conduct primary surveys. Currently, it is essential to include more consistent ecological analyses when the goal is to characterize a subterranean community (Souza-Silva et al. 2011c). Thus, our comparisons were based on Souza-Silva et al. (2011a, 2015) who presented a comprehensive characterization of the invertebrate community structure and threats in the Brazilian Atlantic Forest caves.

The average diversity presented by Souza-Silva et al. (2011a) for the fauna of 91 caves in the Brazilian Atlantic Forest (average = 2.27, sd = 0.63) is similar to the average found for the 15 caves sampled in the state of Espírito Santo (Table 3). However, the average richness presented by the same 91 caves (mean = 43.20 sd = 22.30) was slightly lower than that found in this present study (Table 3). The β diversity (turnover) of the 15 caves of this study were slightly higher than the range presented by Souza-Silva et al. (2011a) (β diversity = 60.19 to 45.58).

Different caves may have considerably different richness and diversity values, even if using similar sampling efforts. According to Ferreira (2005) and Souza-Silva (2008), these differences may be due to several factors, such as the linear extension of the caves, their trophic conditions and the degree of their human interventions.

The β -diversity values and similarities found show large differences in the community composition of 15 caves and reveal that nearby caves do not necessarily have similar communities. As well as the influence of the type of rock in which the cave is located, variations in the physical (microhabitat availability, humidity, etc.) and trophic structures may lead to differences in the composition and distribution of cave invertebrate communities (Christman and Culver 2001, Schneider et al. 2011, Souza-Silva et al. 2011a, b). Thus, the relationship of increased total richness with increasing cave size can probably be related to the increased availability of microhabitats and food resources for invertebrate fauna (Souza-Silva et al. 2011a). Bat communities, producing large guano deposits, are richer and more abundant in larger caves (Brunet and Medellín 2001). Large deposits, in turn, provide food and microhabitats for a greater number of invertebrate species (Pellegrini and Ferreira 2013). It is noteworthy that the productivity in cave environments is an important predictor of increased richness in invertebrate communities (Culver et al. 2006). However, the use of the cave extension as a parameter to predict the invertebrate richness depends directly on the lithology to which the cave is associated (Souza-Silva et al. 2011a). In this study, limestone caves working as an outlier in the general relationship displayed increasing richness with an increase in linear extension, because there is no significant relationship when the Archimedes Pansini cave (300 m) and Limoeiro cave (600 m) were removed from the analysis. Souza-Silva et al. (2011a) also observed this lack of correlation between cave extension and richness in granite caves in the Atlantic forest as a whole.

Environmental impacts on cave fauna

The Casa Branca cave, located in Itaimbé municipality, showed the least similarity when compared to the other caves. This may be due to the impact of the construction of a church within the cave (Fig. 7B). This prevents the presence of species characteristic of natural cave environments and favors the dominance of opportunistic species tolerant of altered environments.

Troglobiont invertebrates can demonstrate tolerance to certain types of changes which may even occur in partially impacted caves (Simon and Buikema 1997). However, there are certain effects that lead to a complete substitution of the groups that would be expected to be found in such caves. It is important to emphasize that similarity comparisons do not necessarily provide indications of direct impacts: the only effective way to detect the existence of alterations would be to have had the opportunity to know the fauna of these caves before these alterations could have occurred. Under these circumstances, one should make use of other tools to compare the structure and faunal composition of the caves in order to understand the effects of the observed changes. While not the best “tools”, such comparisons allow the evaluation, even crudely, of how some impacts may alter the conditions of pristine subterranean ecosystems.

Relevance of cave fauna for conservation

When comparing the richness of troglobiont species found in this study with other karst regions in Brazil and around the world (Culver and Sket 2000; Culver and Pipan 2013; Souza-Silva et al. 2011a, 2015), it can be perceived that the caves sampled to date in the state of Espírito Santo are relatively poor, and cannot be considered hotspots for troglobiont species biodiversity (Fig. 10 and Table 2). However, due to their rarity, the independent phylogenetic history and vulnerability to extinction of the troglobiont species, they deserve special conservation measures. Furthermore, the troglaxene and troglophile invertebrate communities appear to be relatively richer in species compared to other caves in Brazil (Souza-Silva et al. 2011a).

Final considerations

Facing the conditions found in the sampled caves up to now, we recommend emergency attention regarding protective measures, the management and conservation of caves of extremely high and high biological importance, with high impact degree and extremely high vulnerability facing the impacts. It is very important to intensify speleological studies and faunistic surveys, since some areas in the Atlantic Forest in Espírito Santo with the potential for cave occurrence have not been intensively studied (Castelo, Vargem Alta, Nova Venécia, municipalities). Reforestation of at least 250 meters of the cave surroundings that have surrounding pastures, using agroforestry models and management plans and/or ecological restoration of the caves that have received human impacts that come from tourism, religious use and implementation of information and awareness plans for users, with the accompaniment of environmental inspectors at times of mass celebrations and holy days, should be undertaken. Finally it is important to stimulate the creation of Private Natural Heritage Reserves (PNHRs) in the surroundings of caves with troglobiont species, preserved natural vegetation or that under recuperation.

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Cave physical attributes influencing the structure of terrestrial invertebrate communities in Neotropics

Matheus Henrique Simões^{1,2}, Marconi Souza-Silva¹, Rodrigo Lopes Ferreira¹

1 Universidade Federal de Lavras, Departamento de Biologia, Setor de Zoologia Geral, Centro de Estudos em Biologia Subterrânea, Mailbox 3037 Campus Universitário, Zip Code 37200-000 Lavras, Minas Gerais, Brazil **2** Universidade Federal de Lavras, Departamento de Biologia, Programa de Pós-Graduação em Ecologia Aplicada, Mailbox 3037, Campus Universitário, Zip Code 37200 000, Lavras, Minas Gerais, Brazil

Corresponding authors: *Marconi Souza-Silva* (marconisilva@dbi.ufla.br); *Rodrigo Lopes Ferreira* (drops@dbi.ufla.br)

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Abstract

The stability of temperature and humidity in caves is well known. However, little is known if higher or lower cave environmental stability (temperature, humidity, light and others) implies changes in the structure of the biological communities. Number, position and size of entrances, then size, depth, host rock and extent of the cave, the amount and type of food resources are all factors that can have strong influence on the cave biological communities. The objective of the present study was to evaluate the correlation between the presence of water bodies, size of entrances and the linear development of caves with the terrestrial invertebrate richness and species composition in 55 limestone caves located in the Brazilian Savannah, sampled from 2000 to 2011. Invertebrates were sampled by active search throughout the caves, prioritizing micro-habitats (sites under rocks) and organic resources (litter, twigs, feces and bat guano). We recorded 1,451 invertebrate species. Species richness was positively correlated with presence of cave streams, width of entrances and linear development of the caves. The richness of troglomorphic species was positively correlated to the presence of perennial pools and linear development of the caves. The presence of cave streams was a decisive factor for determining the community structure, increasing the number and the similarity of troglophile species among the caves. Flood pulses can cause disturbances that eventually select the same species besides importing resources. However, for the terrestrial troglomorphic species the disturbance caused by cave streams may decrease the number of species.

Keywords

Cave entrances, linear development, cave streams, puddles, subterranean fauna, invertebrates

Introduction

Caves are usually dark, have constant temperature and high humidity according to the isolation from the surface, thus resulting in high environmental stability (Poulson and White 1969, Culver 1982). The availability of food resources in caves is limited, predominantly with allochthonous organic matter being imported by lotic and percolating water, bats and plant roots (Poulson and White 1969, Simon et al. 2003, Culver and Pipan 2009, Souza-Silva et al. 2011a and 2012).

Subterranean environmental stability is directly related to how isolated it is from the epigeal environment. The number, width, position and distribution of the entrances in relation to the extension of the caves can increase or reduce the environmental stability of the cavity and consequently provoke changes in their biological community structures. Besides influencing environmental stability, these metrics can limit or increase the availability of food resources and likewise influence the number of species colonizing the environment (Ferreira 2004, Souza-Silva et al. 2012a).

Hydrological changes can be another factor that influences the cave fauna. Cave streams and perennial pools can act increasing the humidity and importing organic matter, being determinant for the food resources availability (Souza-Silva et al. 2012 and 2012a).

Differences in species number between distinct places have puzzled naturalists and ecologists and several hypotheses have been developed to explain these differences (Williams 1964, MacArthur and Wilson 1967). In general, species number change according to the temporal and spatial habitats traits.

It is known that the number of troglobitic species increases as the sampled area increases (Culver et al. 1999), as well as the total number of species increases with the size of cave (Culver et al. 2004, Ferreira 2004, Souza-Silva 2008). The cave size also influences the number of species at different levels according to the cave lithology. For example, the increase on the number of species as the cave size increased is more intense in iron core caves when compared to the other lithologies (Souza-Silva et al. 2011b).

Changes in species composition and richness through replacement, loss or gain among different caves of the same area or in the same cave can occur over time and space (Bento 2011, Souza-Silva et al. 2011b, Souza-Silva et al 2012a). Some methods have been proposed to evaluate beta diversity, that consider the degree of change in community composition or the community differentiation, in relation to a gradient of environment, or distinct samples (Whittaker 1960, Baselga 2007, 2010, Carvalho et al. 2012, 2013).

In this paper we verified the influences of cave metrics (width of entrances and linear development) and the presence of water bodies (presence of temporary or perennial puddles and of streams and seasonal flooding) on invertebrate cave fauna of the Neotropical region.

Methods

Study area

We conducted the study from 2000 to 2011 in 55 limestone caves of the Brazilian Savannah, northwest of Minas Gerais state, Brazil (Figure 1). According to climatic classification of Köppen-Geiger, the region is classified as *Aw* with two distinct seasons, rainy and dry. The region presents an average annual temperature between 20 and 26 °C and maximum relative humidity around 80% and minimum around 50% (Alvares 2014).

Cave metrics and water bodies

We measured the width of entrances and linear development of the caves. We considered the width of entrance as the greatest horizontal length of the entrance profile and cave linear development as the linear development sampled in each cave. Some caves were not sampled throughout their total length. We placed the caves in four categories regarding water bodies: permanently dry, with puddles (perennial or seasonal), dry but subject to seasonal flooding, and with perennial cave streams.

Sampling terrestrial cave fauna

Only terrestrial invertebrates were sampled during the study and each cave was visited once. We carried out the sampling by visual searching across the accessible parts of the cave, prioritizing organic deposits (debris, carcasses, guano, etc.) and microhabitats (spaces under rocks, humid soil, cracks, speleothems). Extensive visual searching and manual collections were made with the aid of tweezers, brushes and entomological nets (Ferreira 2004, Souza-Silva et al. 2011b). The collection team was always composed by five biologists (always the same team) with experience in caving and manual collection of invertebrates, as recommended by Weinstein and Slaney (1995). To ensure that the sample was the most standardized as possible, the sampling time was approximately 10 minutes in 10 m² for each biologist (Souza-Silva et al 2011b).

We separated all specimens into morphospecies taxa for all statistical analysis (Oliver and Beattie 1996, Derraik et al. 2002, Ward and Stanley 2004, Derraik et al. 2010, Souza-Silva et al. 2011b). Oliver and Beattie (1996) showed that morphospecies identified by non-specialists can provide estimates of richness and turnover consistent with those generated using species identified by taxonomic specialists. The use of morphospecies or corrected morphospecies inventories in the analyses provided results generally concordant with conventional species inventories (Oliver and Beattie 1996a).

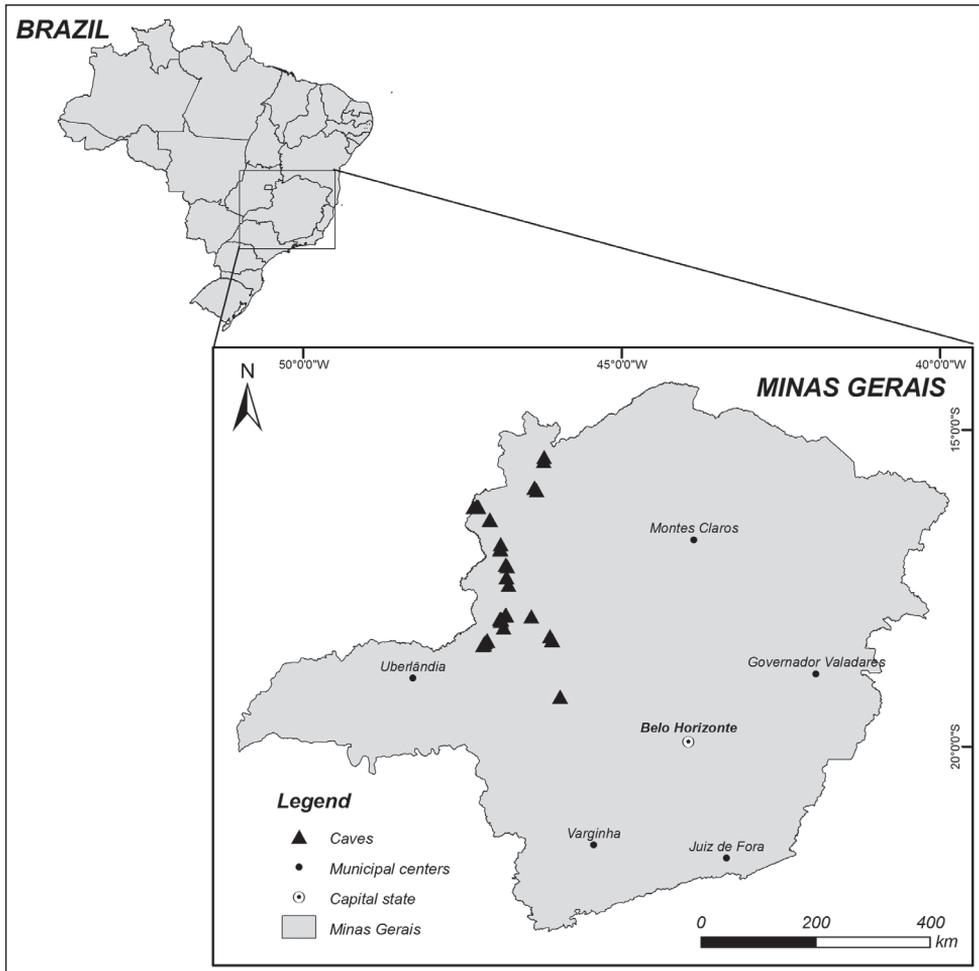


Figure 1. Cave distribution at Minas Gerais state, Brazil (black triangle), where terrestrial invertebrates were sampled.

Determination of troglobite/troglophic species

We determined the troglobite/troglophic species through the identification of troglomorphisms in the specimens. Such characteristics vary among the groups, but frequently are represented by the reduction of melanin pigmentation, reduction of ocular structures and elongation of appendages (Culver and Wilkens 2000, Culver and Pipan 2009).

Statistical analyses

To normalize the variables the values of entrance width and linear development of the caves were log-transformed to reduce the influence of extreme values. The total species

richness, was normally distributed (Shapiro Wilk test = 0.918; $p = 0.001$). The richness of troglomorphic species recorded a lot of zeros, and it was not possible to reach normality for this variable.

We evaluated the influence of the entrances width and cave linear development on the species richness through linear regression. Influences of the presence/absence of different categories of water bodies were evaluated with ANOVA one-way for total richness and non-parametric ANOVA (Kruskal-Wallis test) for richness of troglomorphic species. One of the sampled caves (Deus Me Livre Cave) possesses a singular condition: despite being dry throughout the year, it is subject to seasonal flooding, and was not considered in the analyses.

We used the Jaccard index to compare the fauna composition in different caves (Magurran 2004). This index is the most suitable for presence/absence data since it does not assign weight to the species abundance, such as the Bray-Curtis index commonly used in ecological studies. Beta diversity has been calculated in accordance with the proposals of Carvalho et al. (2012, 2013) and Cardoso et al. (2015), in which it is possible to perform the partition of this measure by means of the contribution of replacement and differences in species richness. We performed the partition of beta diversity using BAT package developed by Cardoso et al. (2015). The objective of this analysis was to assess whether the dissimilarity between the communities was influenced more by replacement than by differences in species richness.

We performed the DistLM test to verify the influence of metric parameters and the presence/absence of different categories of water bodies on species composition of the caves (Anderson 2004). This test shows which variable or variables can influence the fauna composition (McArdle and Anderson 2001). We used non-metric multidimensional scaling (nMDS), based on the Jaccard index, to visualize groups of caves according to the variables that best explained the species composition identified in the DistLM test. We performed the ANOSIM one-way (Jaccard index) analysis to test the significance of the separation of groups (Clarke 1993).

Results

The higher entrance width was recorded for Marcela Cave (125 m; Table 1). Lapa Nova Cave, with 4,000 meters sampled, presented the longest linear development (Table 1). Most of the caves ($n = 38$) were dry throughout the year and others had water bodies. Nine caves had puddles, one was subject to seasonal flooding and seven of them had rivers (Table 1).

We recorded 1,451 invertebrate taxa, distributed in at least 174 families (Table 2). Diptera presented the highest richness (326 taxa), followed by Coleoptera (250 taxa) and Araneae (169 taxa) (Figure 2). Families with the highest number of taxa recorded were Chironomidae (45 taxa), Staphylinidae (79) and Theridiidae (24) respectively (Table 2). The average richness was 58 morfospecies ($SD = 26$). Lapa Nova cave presented the highest richness (153 taxa), followed by the Vereda da Palha cave (107 taxa) (Table 1).

Table 1. Municipalities, caves, water bodies (WB) (CS: cave streams, P: puddles, SF: dry caves subject to seasonal flooding, D: dry), width of entrances (WE), sampled linear development (LD), total number of species (S), number of troglomorphic species/troglobitic (TS) in the studied area.

Municipalities	Caves	WB	WE (m)	LD (m)	S	TS
Arinos	Camila	CS	5	120	98	2
	Capa	CS	17	480	101	0
	Marcela	CS	125	400	78	0
	Suindara	D	16.9	160	56	0
	Salobo	P	6.8	40	47	2
	Taquaril	CS	5	150	70	1
	Velho Juca	D	7.2	70	47	2
Cabeceira Grande	Caidô	D	30	400	71	1
	Porco Espinho	D	4	17	36	0
Coromandel	Huguinho	D	4	35	38	0
	Urubu	D	2	50	34	0
	João do Pó	D	4	180	48	0
	Ronan	D	10	1000	46	0
	Ronan II	D	6.5	160	25	0
	D'água	P	9	80	33	0
	Morcegos	D	3	86	31	0
João Pinheiro	Sapecado	D	1.5	20	26	0
	Tauá	D	15.4	26	22	0
Lagamar	Vendinha	D	7	300	72	0
Matutina	Cachoeira	P	13.3	20	59	0
	Nove	D	1.6	7.85	48	0
	Campo de Futebol	D	15	25	42	0
Paracatu	Lagoa Rica	P	5	200	53	6
	Tamanduá II	D	2	38	41	0
	Cava	D	3.3	38	48	0
	Santa Fé	D	21	78	30	0
	Brocotó	D	4.5	30	72	0
	Brocotó II	D	5	60	73	0
	Santo Antônio	P	13.8	67	51	0
Presidente Olegário	Caieira	D	22	200	61	0
	Juruva	CS	15	250	105	1
	Vereda da Palha	CS	14	250	107	0
Unaí	Abriguinho	D	6.5	8	34	0
	Barth Cave	D	14	160	47	1
	Cachoeira do Queimado	D	52	160	57	2
	Encosta	D	2	40	52	0
	Mata dos Paulista	CS	1.5	30	63	0
	Frangas	D	3	13	41	0
	Deus Me Livre	SF	9	50	106	0
	Rio Preto	D	4.6	38	56	2
	Malhadinha	D	5	70	98	2
	Sapezal	P	15	130	78	0

Municipalities	Caves	WB	WE (m)	LD (m)	S	TS
Vazante	Abrigo da Escarpa	D	10	4	36	0
	Escarpa	D	3	63.3	63	0
	Urtigas	D	30	369	70	2
	Urubus	D	24	61.3	93	3
	Não Cadastrada	D	2	18.4	49	1
	V01	D	2	5	15	0
	V02	D	1.5	10	38	2
	Delza	P	4	1400	46	5
	Mata Velha	P	7	160	61	0
	Guardião Severino	D	15	50	47	0
	Lapa Nova	P	45	4000	153	6
	Lapa Nova II	D	4.5	600	55	3
	Sumidouro da Vaca Morta	D	7	16.1	72	0

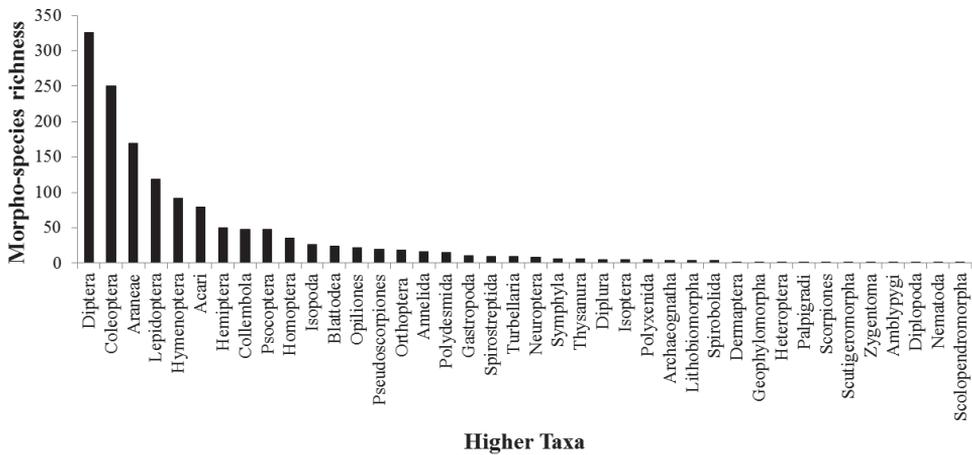


Figure 2. List of sampled higher taxa and their species richness.

Only 2.3% of the invertebrates presented troglomorphic traits (33 taxa), distributed in 18 of the 55 sampled caves. Such taxa included Araneae (eight species), Isopoda (six species), Collembola (six species), Polydesmida (five species), Acari, Hirudinea, Coleoptera, Opiliones, Palpigradi, Polyxenida, Pseudoscorpiones and Turbellaria (one species each) (Table 3). The caves with the highest richness of troglotic species were the Lagoa Rica and Lapa Nova caves, with six species each one.

A significant difference was observed between the total richness of taxa and width of entrances ($R: 0.424$, $p: 0.001$), linear development ($R: 0.519$, $p < 0.001$) and presence of water bodies in the caves ($R^2: 0.279$, $F: 9.876$, $p < 0.001$), and the richness of taxa was higher in caves with rivers (Figure 3).

Table 2. Higher taxa and families recorded in 55 limestone caves in the Brazilian Savannah. Un: unidentified. Species numbers recorded for the families are inside the parentheses.

Higher taxa		Families
Annelida	Oligochaeta	Un
Arachnida	Acari	Ameroseiidae (1), Anoetidae (1), Anystidae (1), Argasidae (2), Bdellidae (3), Cheiletidae (1), Erythraidae (4), Ixodidae (3), Laelapidae (6), Macrochelidae (5), Macronyssidae (4), Melicharidae (1), Ologamasidae (1), Opilioacaridae (1), Oropheidomenidae (1), Parasitidae (1), Phthiracaridae (1), Podocinidae (1), Rhagidiidae (3), Teneriffidae (1), Veigaiidae (2).
	Amblypygi	Phryniidae (1)
	Araneae	Actinopodidae (1), Araneidae (16), Caponiidae (1), Ctenidae (12), Deinopidae (3), Dictynidae (1), Dipluridae (1), Filistatidae (1), Gnaphosidae (1), Leioididae (1), Nemesiidae (2), Ochyroceratidae (2), Oonopidae (12), Palpimanidae (1), Pholcidae (7), Prodidomidae (3), Pisauridae (1), Salticidae (10), Scytodidae (2), Segestriidae (1), Sicariidae (1), Sparassidae (1), Symphytognathidae (2), Tetrablemmidae (1), Tetragnathidae (1), Theraphosidae (1), Theridiidae (24), Theridiosomatidae (2), Trechaleidae (2), Uloboridae (2)
	Opiliones	Gonyleptidae (12), Escadabiidae (2).
	Palpigradi	Eukoeneriidae (2)
	Pseudoscorpiones	Chernetidae (4), Chthoniidae (6), Garypidae (2).
	Scorpiones	Buthidae (1)
	Crustacea	Isopoda
Insecta	Archaeognatha	Meinertellidae (4)
	Blattodea	Blaberidae (1), Blattellidae (15), Blattidae (8)
	Coleoptera	Bostrichidae (1), Carabidae (29), Cholevidae (3), Chrysomelidae (4), Curculionidae (6), Dermestidae (6), Dryopidae (3), Elateridae (9), Elmidae (3), Endomychidae (1), Histeridae (3), Lampyridae (2), Nitidulidae (1), Omophronidae (1), Pselaphidae (9), Ptiliidae (3), Ptylodactylidae (6), Scarabaeidae (6), Staphylinidae (79), Tenebrionidae (16)
	Collembola	Arrhopalitidae (4), Dicyrtomidae (2), Hypogastruridae (1)
	Dermaptera	Labiidae (2)
	Diplura	Japygidae (1)
	Diptera	Agromyzidae (4), Anthomyzidae (1), Asilidae (2), Calliphoridae (1), Cecidomyiidae (36), Ceratopogonidae (15), Chironomidae (45), Chloropidae (1), Culicidae (2), Dixidae (1), Dolichopodidae (7), Drosophilidae (19), Empididae (1), Keroplatidae (1), Lauxaniidae (1), Milichiidae (6), Muscidae (6), Mycetophilidae (12), Phoridae (18), Psychodidae (18), Sarcophagidae (1), Sciaridae (13), Simuliidae (3), Stratiomyidae (5), Streblidae (1), Syrphidae (1), Tabanidae (1), Tipulidae (25)
	Hemiptera	Cydnidae (6), Hebridae (10), Ploiariidae (8), Reduviidae (7), Cicadellidae (17), Cixiidae (12), Thyreocoridae (1)
	Hymenoptera	Apidae (1), Braconidae (1), Eupelmidae (1), Encyrtidae (1), Evaniidae (2), Formicidae (57), Halictidae (1), Ichneumonidae (2), Mutillidae (1), Pteromalidae (2), Vespidae (2)
	Isoptera	Termitidae (3)
	Lepidoptera	Arctiidae (3), Geometridae (2), Hesperidae (3), Noctuidae (24), Pyralidae (7), Satyridae (1), Tineidae (54)
	Neuroptera	Ascalaphidae (1), Mantispidae (1), Myrmeleontidae (5)

Higher taxa		Families
	Orthoptera	Gryllidae (2), Phalangopsidae (3), Tettigoniidae (1)
	Psocoptera 4	Lepidopsocidae (2), Liposcelididae (3), Psyllipsocidae (9), Ptiloneuridae (6)
	Zygentoma 4	Atelurinae (2), Lepidotrichidae (1), Lepismatidae (1), Nicoletiidae (4)
Mollusca	Gastropoda	Un
Myriapoda	Geophilomorpha 1	Geophilidae (2)
	Lithobiomorpha 1	Lithobiidae (1)
	Polydesmida 2	Chelodesmidae (1), Paradoxosomatidae (1)
	Polyxenida	Polyxenidae (5)
	Scolopendromorpha 2	Cryptopidae (1), Scolopendridae (1)
	Scutigermorpha 1	Scutigerae (2)
	Spirobolida 1	Rhinocricidae (1)
	Spirostreptida 1	Pseudonannolenidae (6)
	Symphyla 2	Scolopendrellidae (2), Scutigrellidae (2)
Nematoda	Nematoda	Un
Platyhelminthes	Temnocephalida	Un
Turbellaria	Turbellaria	Un

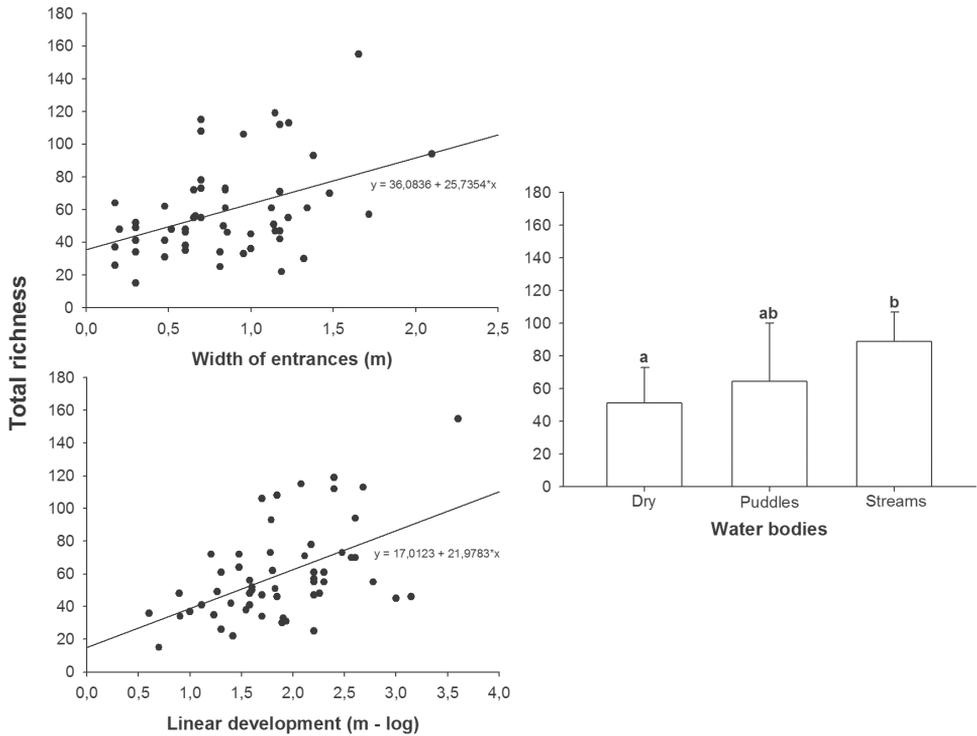


Figure 3. Correlation between total richness and width of entrances, linear development and water body presence/absence. The barr represents the average and the trace the standard deviation. Different letters indicate significant differences in average richness.

Table 3. List of troglomorphic/troglobitic species recorded in the sampled caves in the Brazilian Savannah, Minas Gerais state, Brazil, in the years 2000, 2009, 2010 and 2011. Un: unidentified.

Higher taxa	Family	Morphospecies	Caves
Acari	Un	Trombidiforme sp8	Rio Preto
Annelida	Un	Hirudinea sp3	Salobo
Araneae	Ochyroceratidae	Araneae sp24	Barth cave
		Ochyroceratidae sp1	Urubus cave
	Oonopidae	Oonopidae sp3	Lapa Nova cave
		Oonopidae sp4	Lagoa Rica cave
	Prodidomidae	Prodidomidae sp3	Cachoeira do Queimado cave
		Prodidomidae sp1	Delza cave
	Tetrablemmidae	Tetrablemmidae sp1	Lagoa Rica cave
	Un	Araneae sp17	Não Cadastrada cave
Coleoptera	Pselaphidae	Pselaphidae sp10	Rio Preto
Collembola	Arrhopalitidae	<i>Arrhopalites</i> sp1	Delza, Lapa Nova, Lapa Nova II
	Un	Collembola sp5	V02
	Hypogastruridae	<i>Acherontides</i> sp1	Lapa Nova, Lapa Nova II
	Un	Collembola sp12	Lagoa Rica
	Un	Collembola sp32	Camila
	Un	Collembola sp34	Malhadinha
Isopoda	Platyarthridae	<i>Trichorhina</i> sp1	Lagoa Rica, Urtigas, Delza, Lapa Nova
		<i>Trichorhina</i> sp3	Urubus
		<i>Trichorhina</i> sp5	Camila
		<i>Trichorhina</i> sp.	Velho Juca, Malhadinha
	Styloniscidae	Styloniscidae sp1	Urtigas, Delza
		Styloniscidae sp5	Juruva
Opiliones	Escadabiidae	<i>Spelaeolectes</i> sp1	Lagoa Rica
Palpigradi	Eukoeneiidae	<i>Eukoeneia virgemdalapa</i>	Lapa Nova
Polydesmida	Un	Polydesmoidea sp1	Lapa da Delza
	Un	Polydesmoidea sp2	Lagoa Rica
	Un	Polydesmoidea sp3	Caidô, Cachoeira do Queimado
	Un	Polydesmoidea sp4	Velho Juca
	Un	Polydesmida sp2	Urubus
Polyxenida	Polyxenidae	Polyxenidae sp5	Taquaril
Pseudoscorpiones	Chthoniidae	Chthoniidae sp2	V02
Turbellaria	Un	Turbellaria sp6	Salobo

No significant relation was observed between the richness of troglomorphic species and width of entrances. However, there was a significant relation between the richness of troglomorphic species and the linear development ($R: 0.460$, $p < 0.001$) and presence/absence of water bodies ($H: 4.722$, $p < 0.013$), with higher values in caves with puddles (Figure 4).

In general the faunal troglophile composition was quite dissimilar between the caves (average $B_{total}: 0.9786$; variance: 0.0007). The recorded dissimilarity is explained

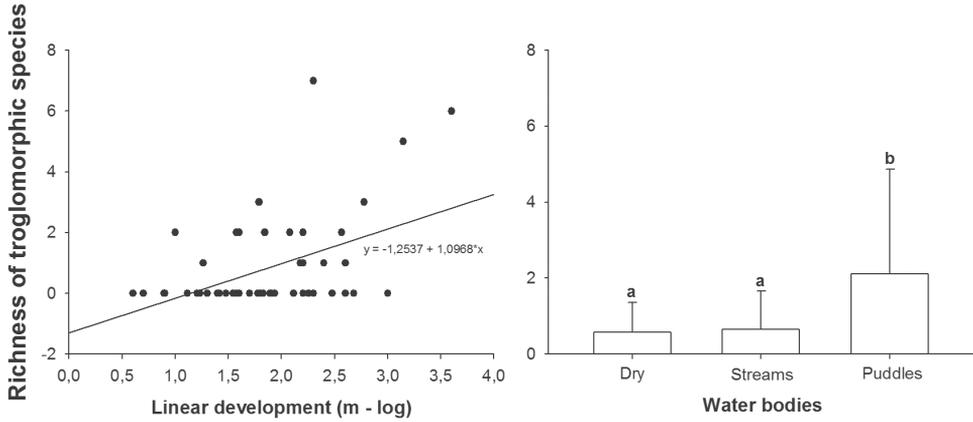


Figure 4. Correlation between the richness of troglomorphic species and linear development and water body presence/absence. The barr represents the average and the trace the standard deviation. Different letters indicate significant differences in average richness.

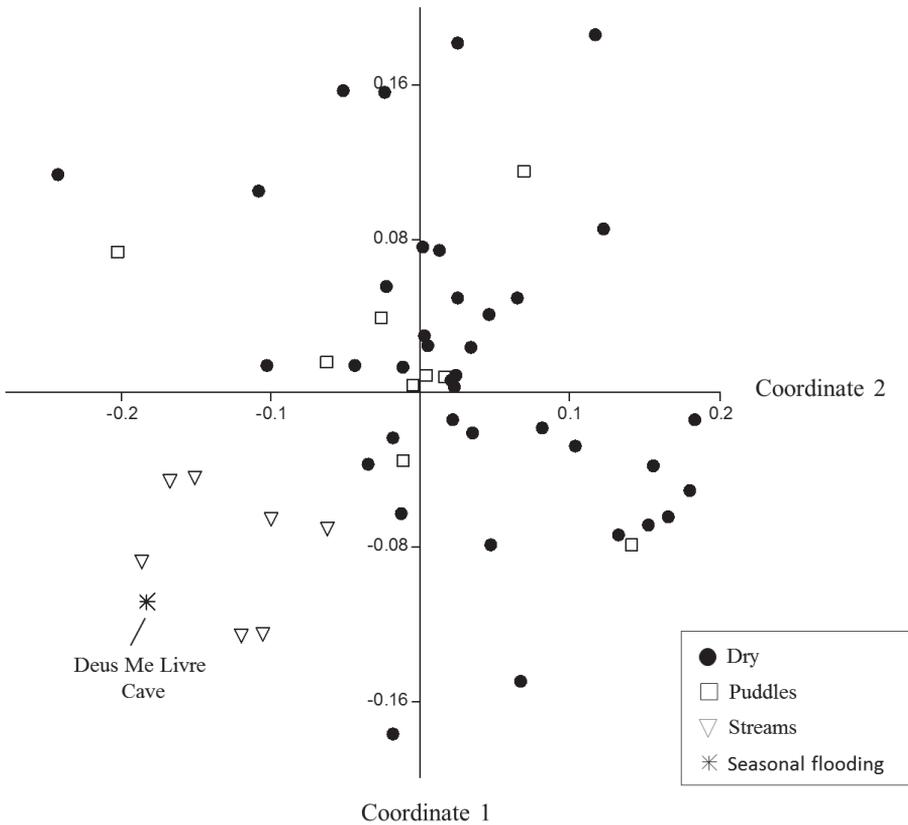


Figure 5. Non-metric multidimensional scaling (Jaccard index) using presence and absence of species sampled in 55 limestone caves of the Brazilian Savannah. The figure shows that the cave, despite dry most of the year, is subject to seasonal flooding (Deus Me Livre cave), and then was more similar to caves with streams.

by the replacement of species (B_{repl} : 0.9786705). The contribution of differences between number of species is near-zero ($B_{\text{rich}} < 0.0000001$).

Despite the general high dissimilarity, the presence of water bodies significantly influenced the species composition (DistLM Test, Pseudo-F: 1.901, R^2 : 0.054, $p < 0.001$). The non-metric multidimensional scaling analysis (nMDS) showed that among the water body categories, cave with streams were more similar regarding the faunal composition (Figure 5, Stress: 0.18). This separation was confirmed by ANOSIM (one-way). A significant difference was observed between caves with streams and dry caves (R : 0.443, $p < 0.001$) and caves with streams and with puddles (R : 0.541, p : 0.002), while dry caves and caves with puddles were not significantly different.

Discussion

Little is known about the effects of physical characteristics determining the cave community richness and composition. Most of the studies regarding this topic showed that number of species increases in large caves and with more entrances (Culver et al. 2003, Culver et al. 2004, Ferreira 2004, Souza-Silva et al. 2011b, Souza-Silva et al. 2012). Corroborating these previous studies our results demonstrated the effects of the cave metric parameters on the number of terrestrial invertebrate species associated to limestone caves in Brazil. Regarding the influence of the presence of water bodies into the caves, our findings are new since no previous studies have shown similar results.

The relation observed between width of entrances and number of species (Figure 3) can be due to the fact that large entrances probably function as “windows” that facilitate the colonization of hypogean systems by external invertebrates as well as the input of organic matter. Caves with large entrances may have more interface areas with the surrounding epigeal system, thus increasing the establishment of para-epigeal communities (Ferreira and Martins 2001, Prous et al. 2004). It is worth noting that caves with more entrances potentially may be capable to receive a greater amount of organic material from the epigeal environment, then increasing the food resource availability inside the caves.

It is valid to note that the tropical region presents external conditions milder than those observed in temperate climate regions. Entrances of tropical caves provide excellent shelter sites and even permanence for several species (Prous et al. 2004), different from what occurs in many temperate caves, in which the entrances, especially in the winter, are almost as severely affected by the cold as the external environment (Culver and Pipan 2009).

The increase in the linear development of caves was related to total number of taxa (Figure 3) and number of troglobitic species (Figure 4). This tendency was also observed in previous studies (Culver et al. 2003, Culver et al. 2004, Ferreira 2004, Souza-Silva et al. 2011a). Larger caves present higher habitat and resource availability, which are decisive factors for the subterranean fauna (Culver et al. 2006), thus allowing higher number of species to establish (Culver et al. 2004, Ferreira 2004, Souza-Silva 2008). As

an example, one can mention that larger caves allow the establishment of more species and larger populations of bats (Brunet and Medelin 2001), then increasing the productivity of guano. Adding our findings to the above mentioned studies we can say that there is a positive relationship among the linear development, availability and variety of habitats, resource availability and the number of species colonizing the cave environment. However, these variables work together and can influence in different ways and levels. One example is that a cave with a linear development of 200 m (Lagoa Rica) has a similar number of total species (53 species) to Delza cave, with a linear development of 1400 m (46 species) and a similar number of troglobitic species (6 and 5, respectively).

Lotic systems, besides increasing the humidity, import organic matter from the surrounding epigeal environment to the inner parts of the caves. This provides food resources for the fauna (Poulson and Lavoie 2001, Souza-Silva et al. 2011a).

Caves are oligotrophic environments and the increasing resource availability allows more species to colonize and remain (Schneider et al. 2001). The amount of organic matter imported by cave streams changes depending on the season, with larger amounts during the rainy period (Souza-Silva et al. 2011a, 2012). Furthermore, during the rains many invertebrate species can be carried into the caves and, since they use organic matter as food and shelter, many species can remain throughout the year, thus increasing the local richness (Souza Silva et al. 2012).

Streams can cause disturbances in the caves, mainly during the rainy period (floods), leading to changes in the cave community (Souza-Silva et al. 2011). These disturbances are comparable to those predicted by the Flood pulse concept, initially proposed for flood plains (Junk et al. 1989). This theory predicts that the system responds in function of the range, duration, frequency and regularity of the pulses. Regular pulses (that can be the case of cave streams) lead terrestrial species to adapt to the conditions of the aquatic/terrestrial transition zones. Furthermore, regular flood pulses can prevent all of the ecological succession stages, as well as may lead to competitive exclusion.

Despite of the stress caused by flood pulses, cave streams maintain high species diversity, similar to what occurs in aquatic/terrestrial zones in flooded plains, a fact that corroborates the intermediate disturbance hypothesis (Connell 1978). It is important to emphasize that temperate and tropical areas will respond differently to the flood pulses and that the flow rate of the cave stream is also a decisive factor (Tockner et al. 2000).

The number of troglobitic species was higher in caves with puddles (Figure 4). Terrestrial invertebrates more specialized to the cave environment (troglobitic) present adaptations to live under extreme moist conditions, as, for instance, cuticular reduction that increases the tegument permeability (Culver 1982). If the permeability of the cuticle is increased, the terrestrial troglobitic are sensitive to low humidity levels, losing water quickly (Howarth 1980). Therefore, the higher richness of troglobitic species recorded in the caves with puddles poses a new question: if the presence of rivers maintains high humidity throughout the year and increase the availability of trophic resources, why do those caves did not present more terrestrial troglobitic species?

Cave streams, in spite of maintaining the high humidity and increasing the availability of resources (Souza-Silva et al. 2011a), can cause disturbances that may eventually

prevent (or make difficult) the emergence of troglobitic species. Caves that undergo violent floods usually do not present many troglobitic species (Elliott 2004). One hypothesis is that the disturbance caused by cave streams can cause constant exchange of terrestrial specimens carried by streams and, consequently, increase the genetic flow, decreasing the occurrence of speciation. This hypothesis still needs to be tested.

One of the main physiological adaptations of the troglobites is the resistance to starvation, and such organisms are more resistant to oligotrophic environments than non-troglobitic species (Huppopp 2012). In caves without streams with low availability of resources the troglobitic species are certainly the best competitors. However, in caves with high availability of food resources in association with the presence of epigeal species, this high availability of food may indirectly be a serious threat to troglobites (Sket 1977). Cave streams can also increase competition, especially in small caves, since more species will be brought from the external environments.

The largest number of terrestrial troglobitic species in caves with puddles indicate that these organisms are specialized to live in places with high humidity, but the disturbance caused by the presence of cave streams can eventually decrease the chances of troglobitic species to emerge. It is important to emphasize that there are exceptions, especially considering caves with large extensions. Such environments can allow distinct species to escape to areas out of the river and such big subterranean extensions certainly “filters” external fauna that could be brought during flooding pulses. However, in small caves with streams and few dry channels, terrestrial species can be severely affected and the troglobitic richness can decrease.

Beta diversity among the caves was high. The contributing factor was the replacement of species and the differences in species richness was near-zero. As we have recorded, the richness of terrestrial species is influenced by the area of the cave, size of the entrances and presence of water. These added parameters can generate strong and unique environmental filters within each cave, making it a highly heterogeneous environment. These can be some of the variables responsible for the high turnover of species between the caves. One may also consider that in tropics high values of beta diversity are expected when compared to temperate regions (Koleff et al. 2003). While in the epigeal temperate regions the turnover of species suffers strong environmental influence, tropics seem to suffer more influence of spatial variations that can limit the dispersal (Myers et al. 2013). Furthermore the turnover can be higher in caves when compared to epigeal areas (Cardoso 2012).

All factors here seen lead us to expect high beta diversity values. This confirms the prior predictions that high degree of micro-endemism occurs among subterranean groups (White and Culver 2012). It is important to mention that we only assessed the diversity of taxa. Considering other types of diversity such as phylogenetic and functional diversity, one would expect other still hidden patterns of diversity to emerge (Cardoso et al. 2014).

Despite the general high dissimilarity, the presence of cave streams influenced the species composition (Figure 5). This influence can be explained by two factors: *i*) the carried organic resource is similar and *ii*) the flood pulse selects the same species that are

carried into the caves (turnover). Visually, most of the organic matter carried into the caves was composed of plant debris (leaves and branches). Resources of similar origin are exploited by similar cave communities (Schneider et al. 2011), in this case, mainly detritivores. Flood pulses can carry soil species together with the organic matter (Souza-Silva et al. 2012a) and only those ones adapted to the floods and the cave environment can survive. Thus, eventually the same species have been selected in different caves.

An example is the Deus Me Livre cave. Despite it is dry during part of the year, it is subject to seasonal flooding caused by runoff during the rainy season, since its entrance is located in the bottom of a sinkhole. The fauna of this cave is more similar to the caves with streams (Figure 5), demonstrating that flood pulses caused by runoff are probably selecting the same species, supporting the previous hypothesis.

In the different Brazilian regions, the litter invertebrate fauna is composed mainly of Acari, Coleoptera, Gastropoda, Oligochaeta, Isopoda, Arachnida, Diplopoda, Chilopoda and Blattaria (e.g. Ferreira and Marques 1998, Moreira et al. 2006). These are also the main groups recorded in caves (Pinto-da-Rocha 1995, Romero 2009), what makes high the similarity between groups of cave invertebrates (especially those with streams) and soil invertebrate fauna in the Tropical region.

Another important factor is that the separation of the species according to the level of association with the cave is not always so simple (for details see Sket 2008), demanding a deep knowledge of the biology of each group, as well as of their presence (or not) in the epigeal systems. Novak et al. (2012), in a study on species distribution in the cave environment, separated the groups in only two categories, troglobitic and non-troglobitic (including all other categories), precisely because of the difficulty on separating the other categories (troglophile, troglone and accidental).

Due to the high similarity between the litter and cave fauna and the difficulty on accurately separate which species are associated to the cave, how can we actually separate the cave fauna from the soil fauna in Neotropics? Many species carried by streams with the organic matter can contain accidental groups, although a lot of species has certainly shown to be pre-adapted to the subterranean systems. Even though these species may use the carried organic matter as shelter and food resource (Souza-Silva et al. 2012a), only with more detailed long term studies it will be possible to determine the degree of association of those species with the cave environment.

The highlight in this study is the increase in the terrestrial species richness according to metric parameters and the presence of streams, since largest entrances and water courses can influence cave colonization and detritus input. The input of organic matter by streams is important for the maintenance of cave fauna, serving as shelter and food for several species. Caves with puddles presented higher richness of terrestrial troglobites indicating that the humidity maintenance throughout the year is an important factor for the evolution and maintenance of these species. The beta diversity was high among caves, thus indicating physical and environmental heterogeneity that may be unique to each cave. Our findings highlight that big and wet caves shelter more diverse and complex terrestrial invertebrate communities, what enhances the need for conservation, management and restoration of the cave surroundings in tropical caves.

Acknowledgements

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Groping through the black box of variability: An integrative taxonomic and nomenclatural re-evaluation of *Zospeum isselianum* Pollonera, 1887 and allied species using new imaging technology (Nano-CT, SEM), conchological, histological and molecular data (Ellobioidea, Carychiidae)

Adrienne Jochum¹, Rajko Slapnik^{2,10}, Annette Klussmann-Kolb³,
Barna Páll-Gergely⁴, Marian Kampschulte⁵, Gunhild Martels⁶,
Marko Vrabec⁷, Claudia Nesselhauf⁸, Alexander M. Weigand^{9,10,11}

1 Naturhistorisches Museum der Burgergemeinde Bern, Bernastr. 15, CH-3005 Bern, Switzerland **2** Institute of Ecology and Evolution, University of Bern, Baltzerstrasse 6, CH-3012 Bern, Switzerland. **2** Drnovškova pot 2, Mekinje, 1240 Kamnik, Slovenia **3** Zoologisches Forschungsmuseum Alexander König, Adenauerallee 160, 53113 Bonn, Germany **4** Department of Biology, Shinshu University, Matsumoto 390-8621, Japan **5** Universitätsklinikum Giessen und Marburg GmbH-Standort Giessen, Center for Radiology, Dept. of Radiology, Klinik-Str. 33, 35385 Giessen, Germany **6** Department of Experimental Radiology, Justus-Liebig University Giessen, Biomedical Research Center Seltersberg (BFS), Schubertstrasse 81, 35392 Giessen, Germany **7** Department of Geology, Faculty of Natural Sciences and Engineering, Aškerčeva 12, University of Ljubljana, 1000 Ljubljana, Slovenia **8** Department of Phylogeny and Systematics, Institute for Ecology, Evolution and Diversity, Biosciences, Goethe-University Frankfurt, Max-von-Laue Straße 13, 60438 Frankfurt am Main, Germany **9** Department of Animal Ecology, Evolution and Biodiversity, Ruhr University Bochum, Universitätsstraße 150, 44801 Bochum, Germany **10** Croatian Biospeleological Society, Demetrova 1, 10000 Zagreb, Croatia **11** Aquatic Ecosystem Research, University Duisburg-Essen, Universitätsstraße 5, 45141 Essen, Germany

Corresponding author: *Adrienne Jochum* (adrienne.jochum@googlemail.com)

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We especially wish to dedicate this work to the Slovenian malacologist, Jože Bole in commemoration of the 20th anniversary of his death (December 25, 1995). His immense contributions to malacology, especially in *Zospeum* research, have provided the knowledge base upon which we rely today.

Abstract

The minute troglobitic species, *Zospeum isselianum* Pollonera, 1887 (Eupulmonata: Ellobioidea, Carychiidae) is widely distributed within its Southern Alpine-Dinaric range. Its broad distribution and highly variable shell has caused this species to be historically lumped into its current taxonomic state of ambiguity. In an integrative taxonomic approach, phenotypic and genotypic data are synthesized to assess the intraspecific variability recently inferred for this taxon. We collected 16 *Zospeum* specimens in the Slovenian Alpine Arc encompassing the type locality for *Z. isselianum*. The material comprises five morphologically recognized (sub)species. The species are re-evaluated using SEM, X-ray nanotomography (nano-CT), conchological, histological and molecular data.

Four well-defined lineages are present, which can be attributed to i) *Z. isselianum* s.str. from its new type locality (Turjeva jama), ii) a highly morphologically variable lineage that so far cannot be attributed to a single morphospecies, iii) *Z. kupitzense* A. Stummer, 1984 (raised to species rank) and iv) a lineage comprising the two subspecies *Z. alpestre alpestre* (Freyer, 1855) and *Z. alpestre bolei* Slapnik, 1991 plus *Z. isselianum* individuals. The latter is treated as a single taxon *Z. alpestre*. After considering the severely degraded syntype material of *Zospeum isselianum*, we provide a taxonomic re-description and propose a neotype for this species. Furthermore, new diagnostic information is revealed regarding the columella of *Zospeum isselianum* and allied species. Detailed anatomical study reveals new structural aspects of *Zospeum* morphology and provides groundwork for future investigations.

Keywords

Neotypification, lumped species, subterranean microgastropods, Byne's degradation, species flock

Introduction

Systematic research is undergoing a major transition in the assortment of tools and terminology now available in today's taxonomic toolbox. In addition, species delimitation, the practice of recognizing and determining species boundaries, has expanded to evaluate the current inflation of species concepts generated to infer biological information (de Queiroz 2000, Katz et al. 2015). Speciation is an ongoing process influencing traits at varying temporal scales. In assessing subterranean biodiversity, familiar concepts generally used to describe speciation in metapopulations (i.e. monophyly, diagnostic morphology, distinct ecology, reproductive isolation etc.) are not directly practicable. Morphological stasis or overlapping intra- and interspecific character variability often confuse interpretation of species (hypotheses) and confound conventional species delimitation approaches (de Queiroz 2007, Katz et al. 2015). In our case with subterranean ellobioid microsnails, integrative taxonomy provides the most tangible answer to this problem since it pulls together data derived from different data sources including comparative morphology, genetics, ecology and geology to enable inferences about founded, but sketchy species hypotheses. In addition, new imaging technology in conjunction with molecular information opens new perspectives for interpretation, description and definition of morphology (Stoev et al. 2013).

In this work, we conduct the requisite transition from molecular species delimitation approaches (Weigand et al. 2011, 2013) to species clarification in a geographi-

cally compact group of taxonomically challenging troglobitic microgastropods. We test the efficacy of integrative taxonomy using novel imaging techniques to address the large degree of conchological variability in *Zospeum isselianum*, a taxonomic orphan of frequent synonymies and lumped species designations within the Carychiidae (Pollonera 1905, Stossich 1899, Absolon 1916, Kušcer 1932, Alzona 1971, Bole 1974, Giusti and Pezzoli 1982). Geographically proximal congeners to the type locality of *Z. isselianum* are assessed in a molecular and conchological context. This work augments the initial studies of Giusti (1975) and De Mattia (2003) and presents the first comprehensive anatomical investigation using a contemporary framework for *Zospeum* Bourguignat, 1856. Since recent work on subterranean microgastropods using X-ray nanotomography (nano-CT) and scanning electron microscopy (SEM) effectively differentiated genera of small hypselostomatid snails (see Jochum and Malkowsky 2013, Jochum et al. 2014) and highlighted interspecific variation within shells of carychiid morphospecies (Jochum et al. 2013, 2015b), we apply the same imaging approach in this study.

Zospeum is known to inhabit caves of Northern Spain to the Balkan Dinarides. Most species were described in the latter half of the 19th and 20th Centuries based on shell characters such as whorl number, aperture dentition, and shell size as well as shape and number of lamellae circumscribing the columella (Kobelt 1901, Gittenberger 1973, 1980, Bole 1974, Maier 1982 unpublished data, Slapnik 1991). Twenty-four species and ten subspecies have been described (Bank 2013, Weigand 2013, Weigand et al. 2014, Jochum et al. 2015a). Ongoing research however, questions the validity of many of these species designations and simultaneously points to an even greater number of cryptic species. Contrary to the extensive work of Bole (1974), aspects of phenotypic variability and the availability of enough *Zospeum* material for comparison were seldom issues during earlier taxonomic eras. An additional impediment includes the fact, that many shells in historical collections were often culled from river debris and likely originated from populations inhabiting any number of caves located distances away from the specific river, town or cave system considered to be the official type locality.

Six anatomical studies provide the current knowledge upon which morphological findings of this investigation are based (Bole 1974, Giusti 1975, Maier 1982 unpublished data, De Mattia 2003, Martins 2007, Dörge 2010, unpublished data). Weigand et al. (2011) conducted the first molecular analysis (DNA barcoding) of worldwide Carychiidae, including seven Dinaric *Zospeum* morphospecies. In this work, high intraspecific variation was reported for *Zospeum* collected in different caves while a high incidence of cryptic allopatric speciation for several taxa was uncovered. A subsequent study evaluated original species assignments (Weigand et al. 2013). These authors not only found many morphologically unrecognized evolutionary lineages within this taxon, but also revealed that several alleged *Zospeum* species are ambiguously classified products of past taxonomic lumping traditions. However, in light of this past work, consideration of historical designations and recent investigations cannot ignore the fact that conventional molecular genetic methods, on the one hand, have not only solved

many taxonomic conundrums in biological systematics, but have, on the other, also confounded the situation by uncovering more complicated patterns of unrecognized genetic variability, which otherwise, may never have been detected (Sauer and Hausdorf 2012, Jörger and Schrödl 2013, Duda et al. 2014, for detailed discussion of cryptic speciation see Jörger and Schrödl 2013). Still, and in all due respect of this work, we strive for clarity and understanding of these microgastropods: for accuracy in biodiversity studies, for basic biogeographical investigations and for forming conservation strategies.

Past anatomical investigations have involved intrepid and remarkable dissections of some now dubious, *Zospeum* designations (Bole 1974, Giusti 1975, De Mattia 2003). Giusti (1975) conducted the first anatomical investigations on *Zospeum spelaeum* (Rossmässler, 1839) and *Zospeum tellinii* Pollonera, 1889. De Mattia (2003) specifically compared the genital anatomy of *Zospeum spelaeum* from six populations from different caves and one population of *Z. isselianum* (Grotta Nuova di Villanova) from the widely cavernous region of Trieste. These investigations revealed no substantial differences in morphology between and within different populations. Bole (1974) anatomically examined four different species and subspecies of *Zospeum*, emphasizing that his findings provided nothing taxonomically remarkable. Additional interpretation has remained limited due to the difficulties of collecting enough live material to compare different species. Other investigators such as Maier (1982, unpublished data) described *Z. spelaeum schmidtii* (Frauenfeld, 1854) using serial cross sections to reconstruct the anatomy. Dörge (2010, unpublished data) performed sketchy 3D reconstructions to depict the anatomy from stacked histological serial sections for a *Zospeum* species from Konečka zijalka (Kamnik-Savinja Alps, Slovenia). In regards to *Z. isselianum* here, we question the current value of these investigations for *Z. isselianum* species assignments. In light of these anatomical investigations and regarding our proposal to the International Commission on Zoological Nomenclature (ICZN Art. 81) for designating a neotype (Art. 75.5) for *Z. isselianum*, we present here the first perspective of specific organ structures for *Zospeum* using histology in conjunction with shell analysis of geographically proximal allied species and molecular data. For diagrammatic representation, consult Giusti (1975, figs 1, 2) and De Mattia (2003, figs 25, 26).

Zospeum isselianum has up to now, represented a Southern Alpine and Dinaric taxon (Pollonera 1887, 1905, Stossich 1899, Alzona 1971, Bole 1974, Giusti and Pezzoli 1982). It is known to inhabit caves from a few meters above sea level to an altitude of 2000 m (Hamann 1896, Zilch and Jaekel 1962, Bole 1974, Pezzoli 1992, Slapnik 1991, 1994, Slapnik and Ozimec 2004). An excursion through the literature shows this species has been found in: 17 caves in Italy (Pollonera 1887, Alzona 1971, Maier 1982, unpublished data); five caves and one sediment sample in Austria (Maier and Bole 1975, Mildner 1976, Maier 1982, unpublished data); 169 caves, 16 springs and 37 sediment samples from river basins in Slovenia (Kuščer 1925, Bole 1960, 1974, Maier and Bole 1975, Slapnik 1991, 1994, 2001); 70 caves, five springs and one river basin sediment sample from Croatia (Brusina 1870, Langhoffer 1912, 1915a, 1915b, Slapnik and Ozimec 2004) and in seven caves in Bosnia and Herzegovina (Bole 1974). In light of our current reconsideration of earlier species designations and the requisite



Figure 1. *Zospeum isselianum* potential distribution collated from the literature and museum collections (I. Sajko, CSR SASA).

aspects of cryptic speciation in these enigmatic snails, this tallies to 312 potential, but highly questionable localities for *Zospeum isselianum* (Fig. 1). The localities included in the map (Fig. 1) are listed in Suppl. material 1.

Material and methods

Material is housed in the following collections:

CBSS	Croatian Biospeleological Society, Zagreb, Croatia
CSR SASA	Centre for Scientific Research of the Slovenian Academy of Sciences and Arts in Ljubljana, Slovenia
MSNG	Museo Civico di Storia Naturale »Giacomo Doria«, Genoa, Italy
MHNG	Museum d'Histoire Naturelle de Genève, Geneva, Switzerland
MZUT	Museo Regionale di Scienze Naturali Sezione di Zoologia, Torino, Italy
NHMW	Naturhistorisches Museum Wien, Vienna, Austria
NMBE	Naturhistorisches Museum der Burgergemeinde Bern, Bern, Switzerland
RS	Rajko Slapnik malacological collection, Kamnik, Slovenia
SMF	Senckenberg Forschungsinstitut und Naturmuseum, Frankfurt am Main, Germany
SMNH	Slovenian Museum of Natural History Ljubljana, Slovenia

Taxon sampling

Material was collected from caves of the Julian Alps (Turjeva jama) and the Kamnik-Savinja Alps of Slovenia, which represent the southeastern-most extension of the Alpine topographic chain at the transition to the Dinarides (Fig. 1, Table 1). The two ranges cover about 3000 km² and are located in northwestern Slovenia on the border with Italy and Austria (Figs 1, 3). The highest peaks scale to 2800 m in elevation, producing between 500 and 2000 m of topographic relief.

Table 1. Overview of cave locality information. For each of the seven cave localities the investigated specimens, their museum ID, latitude and longitude data, date of collection, elevation and a measurement of the ambient cave temperature are given. Leg. Rajko Slapnik. Museum ID's refer to CSR SASA, RS, or SMNH collections.

Cave locality	Specimen ID (museum ID)	Lat.	Long.	Date	Elevation [m]	air T [°C]
Turjeva jama, Robič, Kobarid, Slovenia (region locus typicus)	1 (37013)	46.2435	13.5046	19.10.2007	253	9.3 (on 01.06.2013)
	2 (37013a)			19.10.2007		
	3 (RS0063a)			01.06.2013		
Ložekarjeva jama, Olševa mountain, Kamnik-Savinja Alps, Slovenia	4 (38474a) (MC SMNH 3291)	46.4268	14.6240	28.09.2009	1050	7.9 (on 28.09.2009)
	5 (38474a)			28.09.2009		
Konečka zijalka, Šmihel nad Mozirjem, Mozirje, Kamnik-Savinja Alps, Slovenia	6 (37698a) (21675)	46.4024	14.9393	16.08.2008	820	7.5 (on 16.08.2008)
	7 (37698a)			16.08.2008		
	8 (37698a)			16.08.2008		
Potočka zijalka, Olševa mountain, Kamnik-Savinja Alps, Slovenia	9 (40600a-1) (RS0059)	46.4493	14.6693	08.06.2012	1630	5.5 (on 08.06.2012)
	10 (40600a-2)			08.06.2012		
Jama na Zgornjih Brsnikih (= Jama pod Farjevim plazom, Jama pod Mokrico), Mokrica mountain, Kamniška Bistrica, Kamnik-Savinja Alps, Slovenia	11 (39948a) (MC SMNH 2216)	46.3093	14.5832	05.06.2001	980	6.0–10.2 (in 1997)
	12 (39948a)			05.06.2001		
	13 (39948a)			05.06.2001		
Tomazičeva zijalka, Podvolovljek, Kamnik-Savinja Alps, Slovenia (locus typicus)	14 (40596a-1)	46.3134	14.6982	30.04.2012	590	8.9 (on 30.04.2012)
	15 (40596a-2)			30.04.2012		
Kamniška jama, Zeleniške Špice, Kamniška Bistrica, Kamnik-Savinja Alps, Slovenia	16 (40595a-1)	46.3386	14.6124	01.05.2012	1400	5.1–6.1 (between 01.05.2003–01.05.2004)
Ihanščica cave, Ihan, Ljubljana, Slovenia	30014, 30042 (RS0103; RS0104)	46.1216	14.6476	17.5.1969 11.10.2013	415	Not recorded

Geologically, the Slovenian Alps belong to the Southern Alpine thrust-belt, which formed during the last 20 MY and is still tectonically active, as evidenced by considerable seismic activity (Fig. 4) (e.g. Vrabc and Fodor 2006). Karstification-prone, Mesozoic carbonate platform rocks blanket most parts of the range. Present-day vertical karst drainage favors the development of vadose shafts, the prevailing cave type. However, many remnants of phreatically formed horizontal cave systems are also present. These remnants of horizontal cave systems suggest rapid and major neotectonic uplift of the area.

Since historical species designations are geographically very general (i.e. “Carniola” or “Krain” for the Eastern Alpine region including southern Austria and Slovenia) and taxonomic data was recorded during eras of former political boundaries, geographical designations and taxonomic traditions, we have endeavored to collect live material of *Z. isselianum* and allied species as close to where the initial sites of species descriptions may have been. We remark however, that although the subspecies, *Zospeum alpestre likanum* Bole, 1960 could be considered in a broader scope of this investigation, it’s geographic type locality (Gornja Cerovačka pečina, Gračac, Croatia) is located distant from the core, proximal Eastern Alpine sites comprising this study (Fig. 3). Our dataset consists of 16 *Zospeum* specimens (Table 2). These specimens either originate from previous studies (see Weigand et al. 2011, 2013) or were sampled within the scope of this study (Table 2).

Table 2. Overview of the morphological and genetic results. The 16 specimens (#) can be attributed to five morphospecies. The genetic delimitation reveals four clusters (color-coded), with four lineages (L1–L4). Individual BOLD-ID. (<http://www.boldsystems.org/>) of each specimen and a link to the broader study of Weigand et al. (2013) are given. New material of the present study is indicated by an asterisk (*).

Morphospecies	#	Lineage	BOLD-ID	Comments
<i>Z. isselianum</i> Pollonera, 1887	1	L1	BARCA121-10	same as Z6 from Weigand et al. 2013
	2	L1	BARCA213-15*	same as Z6 from Weigand et al. 2013
	3	L1	BARCA214-15*	same as Z6 from Weigand et al. 2013
	11	L3	BARCA122-10	same as Z5 from Weigand et al. 2013
	12	L3	BARCA216-15*	same as Z5 from Weigand et al. 2013
	13	L3	BARCA217-15*	same as Z5 from Weigand et al. 2013
	6	L2	BARCA123-10	same as Z7 from Weigand et al. 2013
	7	L2	BARCA124-10	same as Z7 from Weigand et al. 2013
	8	L2	BARCA215-15*	same as Z7 from Weigand et al. 2013
<i>Z. cf. amoenum</i>	9	L2	BARCA211-13*	same as Z7 from Weigand et al. 2013
	10	L2	BARCA212-13*	same as Z7 from Weigand et al. 2013
<i>Z. alpestre alpestre</i>	16	L3	BARCA218-15*	same as Z5 from Weigand et al. 2013
<i>Z. alpestre bolei</i>	14	L3	BARCA219-15*	same as Z5 from Weigand et al. 2013
	15	L3	BARCA220-15*	same as Z5 from Weigand et al. 2013
<i>Z. alpestre kupitzense</i>	4	L4	BARCA125-10	same as Z1 from Weigand et al. 2013
	5	L4	BARCA126-10	same as Z1 from Weigand et al. 2013

Genetic delimitation

The barcoding region of the cytochrome c oxidase subunit I (COI) gene was amplified for 16 specimens from 7 caves including the morphospecies *Z. isselianum* (9 specimens, 3 caves), *Zospeum* cf. *amoenum* (Frauenfeld, 1856) (2 specimens, 1 cave), *Z. alpestre bolei* (2 specimens, 1 cave), *Z. alpestre alpestre* (1 specimen, 1 cave) and *Z. alpestre kupitzense* (2 specimens, 1 cave) (Table 2).

COI sequences for genetic delimitation were either retrieved from the BOLD project “BARCA” or generated anew within this study (Tab. 2). PCR and sequencing were performed following the protocols given in Weigand et al. (2011) without modifications. The alignment was created using the MAFFT-plugin in Geneious 5.4.7. The G-INS-I option for global homology and less than 200 sequences were used. Removal of the primer sequences for LCO1490 and HCO2198 was achieved by manual 3'- and 5'-trimming. The final alignment had a length of 655 bps. No gaps were present.

The Automatic Barcode Gap Discovery (ABGD) procedure was conducted (Puillandre et al., 2011) in order to semi-automatically detect a barcoding gap, which separates intra- and interspecific genetic diversity. The ABGD web server was used (<http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html>). We tested several combinations of relative gap width (X, from 0.2–2.0), the minimal prior intraspecific divergence (Pmin, from 0.001–0.05) and maximal prior intraspecific divergence (Pmax, 0.03–0.10). The number of bins (Nbins) was 20 and we used a Kimura (K80) TS/TV-ratio of 2.0. Additionally, specimens have been grouped and delineated by the reversed Statistical Parsimony (SP) approach after Hart and Sunday (2007). The approach was performed in TCS 1.21. Clusters were delineated with a connection limit of 99% (statistical probability).

Conchological assessment

To differentiate and compare the geographically proximal species comprising our study, available technology such as X-ray-nanotomography (nano-CT) and SEM are used to access new information from traditional morphological characters (external morphology, radula and sinuosity of the columellar lamella). The position and the degree of sinuosity of the columellar lamella is a widely accepted diagnostic character within the Carychiidae (Strauch 1977, Maier 1982, unpublished data, Bank and Gittenberger 1985, Medaković et al. 1999, Jochum et al. 2015). Bole (1974) and Slapnik (1991) on the other hand, found the parietal lamella and dentition in the aperture taxonomically more significant than the configuration of the columella. Focus here is directed from the inside out without the usual, perforating of windows in the shell and the risk of destruction of valuable material. In this work, X-ray perspectives enable us to reconsider characters for *Zospeum* such as the form of the columella (i.e. cylindrical, clavate, presence/absence of secondary basal dilatation, degree of twisting) as well as the relationship of the columellar lamella to the columella (i.e. degree of inclination,

extended or absent). In addition, SEM captures the superficial structural and textural aspects of the thin *Zospeum* shells.

Shells of three populations of *Zospeum isselianum* from three distinct genetic clusters (see results; L1, L2 and L3) were measured according to Slapnik (1991). This data is presented in Tables 3–4.

In order to assess historical species hypotheses in context here, access to type material housed in museum collections is paramount. Type material whenever possible has been obtained for this study (Figs 2A, G–P, 11A–E).

Histology and light microscopy

The formaldehyde-preserved specimens of conchologically determined (RS) *Zospeum isselianum* (Konečka zijalka) and *Z. isselianum* (Turjeva jama, neotype locality) were dissolved in a solution of 5% acetic acid and 10% formaldehyde to dissolve the shell. The snails were subsequently dehydrated in an increasing ethanol series. Pre-infiltration followed with a 96% ethanol and base liquid solution of Technovit 7100 (50:50 mixture) for two hours and then infiltrated over night following the manufacturer's instructions (Technovit 7100, Heraeus Kulzer GmbH, Wehrheim/Ts., Germany). The specimens were subsequently embedded in cold-polymerizing hydroxyethyl methacrylate resin. Serial sections (1 μm) were prepared using the Leica RM2165 automatic Rotation Microtome (Leica Microsystems Nussloch, Germany), stained with toluidine blue and examined with a high-powered microscope (Leica DM LB2). Histological photomicrographs were taken using a digital camera (Leica DC 300F) and further processed using the software Photoshop 6.0 (Adobe Systems Inc., San Jose, CA, USA).

Scanning Electron Microscopy (SEM)

The radula of *Zospeum isselianum* (Turjeva jama, neotype locality) was prepared according to Holznagel (1998), preserved in 96% ethanol and mounted onto a prepared SEM stub. The radula was sputtered with gold (1–2 x for 60 seconds) in the Agar Sputter Coater (Agar Scientific, Stansted, UK) and viewed in the high vacuum mode of the Hitachi S-4500 Scanning Electron Microscope (15 kV, probe current 20–100 pA) using the secondary electron detector. Photographs were taken with DISS–Digital Image Scanning System 5 (Point Electronic, Halle, Germany).

Shells or preserved full-bodied snails selected for SEM were microscopically evaluated for stability (presence of cracks, shell thickness, evidence of erosion). The sturdiest shells were first wetted in a dish of 80% ethanol or water and then manually brushed clean of cave encrustations using fine, tapered dental brushes, whereby each specimen was gently rotated back and forth between the brushes until it was sediment free. Specimens were then mounted onto double-sided carbon tabs. The samples were sputtered and processed using the same systems as for the radula.

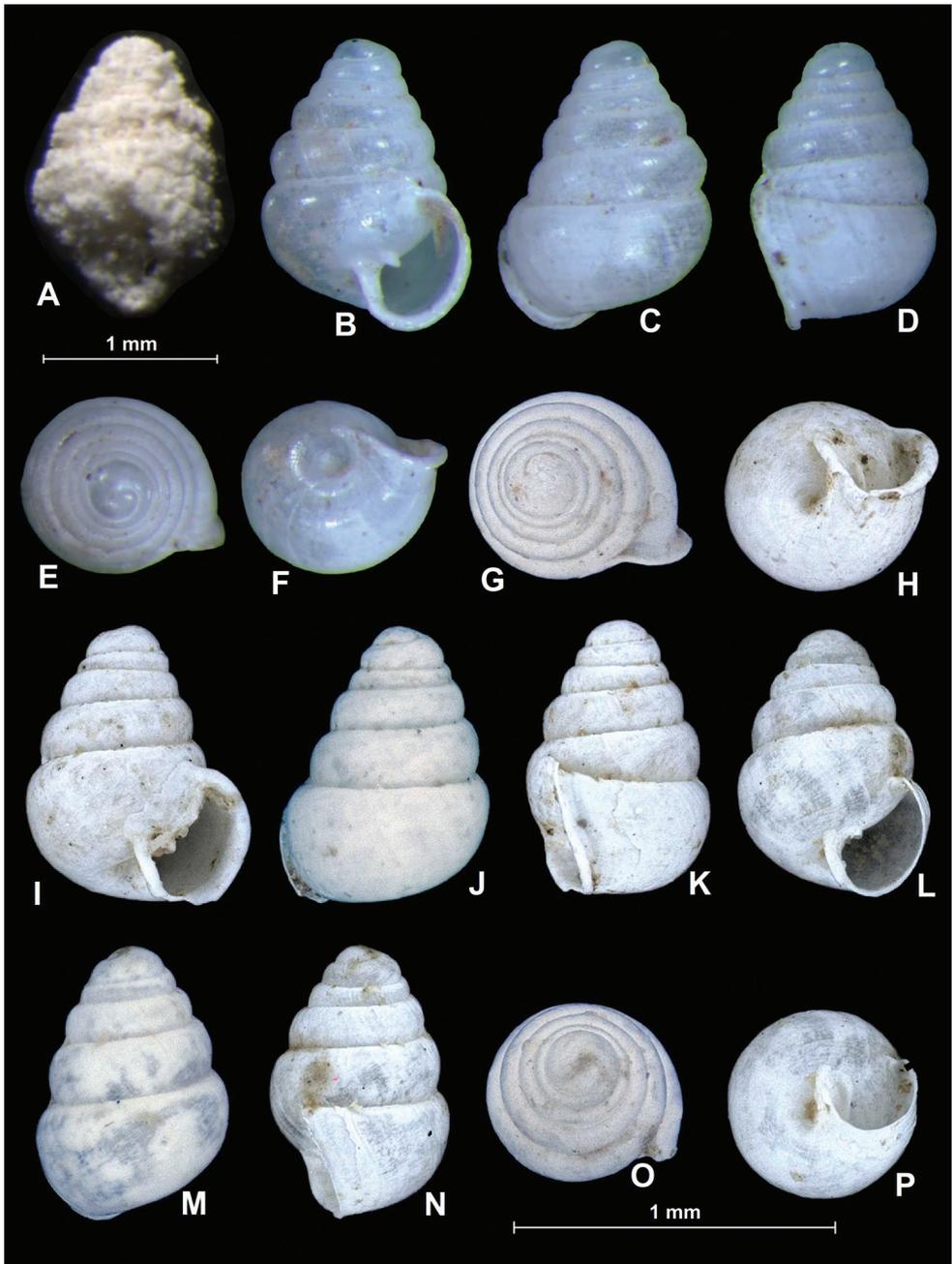


Figure 2. Historical material: **A** *Zospeum isselianum* syntype (MZUT M3232): upper mountains of Natisone River valley **B–F** *Zospeum isselianum* topotype: Turjeva jama **G–P** Specimens labelled as “*Zospeum alpestre*” in the Bourguignat Collection (MHNG 7898/2) from “Cav[erne]. de Carniole”.

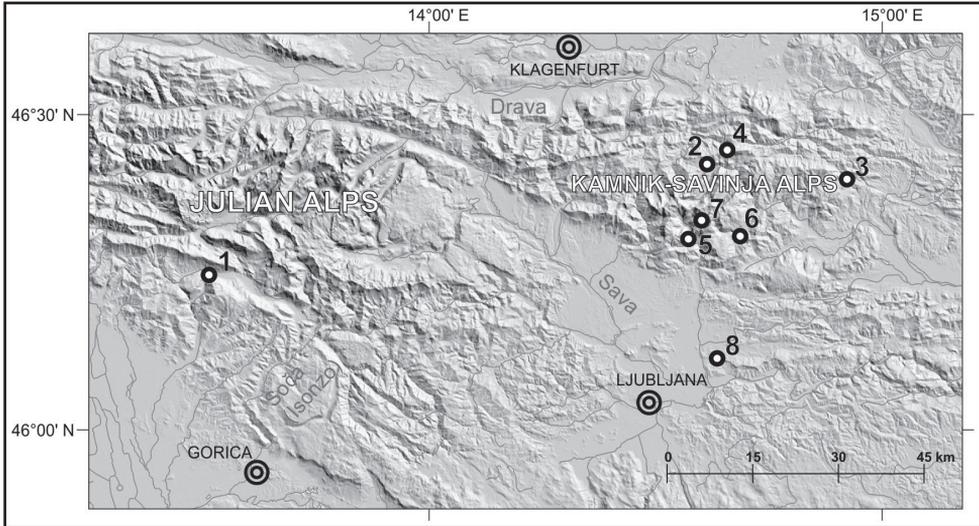


Figure 3. Geographical position and river drainage systems of the Julian Alps and the Kamnik Savinja Alps range. Sampling sites: **1** Turjeva jama **2** Ložekarjeva jama **3** Konečka zijalka **4** Potočka zijalka **5** Jama na Zgornjih Brsnikih **6** Tomažičeva zijalka **7** Kamniška jama **8** Ihanščica cave. Digital terrain model from Jarvis et al., 2008. River network data courtesy of Public Information of Slovenia, the Surveying and Mapping Authority of the Republic of Slovenia, DPK1000V (2008).

X-ray nanotomography

Specimens were imaged using a nano-computed tomography system (nano-CT), manufactured and developed by Bruker-Micro-CT/SkyScan (SkyScan 2011, Kontich, Belgium) at the Department of Experimental Radiology, Justus-Liebig University Biomedical Research Center Seltersberg (BFS), Giessen, Germany. The system contains an open pumped type X-ray source, a LaB₆ cathode and a transmission anode consisting of a tungsten-coated beryllium window. Enhanced edge sharpness and submicron resolution are gained by a high-focused X-ray spot of <400 nm side length (see Langheinrich et al. (2010) for more details). Specimens of *Zospeum isselianum* (Turjeva jama, neotype locality) were mounted on a specimen holder and then fixed on a computer-controlled stage. They were then scanned 185° around their vertical axis in rotation steps of 0.23° at 80 kV tube voltage and 120 µA tube current. Reconstruction of cross sectional images was performed using a Feldkamp cone-beam reconstruction algorithm. Image resolution of the cross sectional images was 1,75 µm isotropic voxel side length with a grey scale resolution of 8 bit. Digital image post processing and visualization (maximum intensity projection MIP, volume compositing and summed voxel projection) were displayed using the ANALYZE software package (ANALYZE 11.0, Mayo Clinic, Rochester, MN, USA).

Digital imaging

Zospeum species were photographed using a Kontron-Elektronik-ProgRes-3012 microscope camera (Jena, Germany) and a Leitz MZ12 stereomicroscope.

Results

Genetic delimitation

The ABGD approach delineates four clusters (Fig. 4): *Z. isselianum* (L1), *Zospeum* sp. (L2), *Z. alpestre* (L3) and *Z. kupitzense* (L4). The SP approach delineates five clusters, thereby splitting up L2 into two clades comprising *Z. isselianum* and *Z. cf. amoenum* specimens only, respectively. The maximal K2P-genetic distance between the two lineages in L2 is 2.3% (Table 3, Figs 5, 6), which is still within the threshold of intraspecific variability and thus, below the barcoding gap of 3.2% calculated for Carychiidae (Weigand et al. 2013). We refer to a more conservative approach here and consider four clusters (i.e. Lineage 1, L2, L3, and L4) whereby, the minimal K2P-interspecific distance in this study is 5.4% (Fig. 7).

	morphology	ABGD	SP	taxon
1	<i>Z. isselianum</i>	█	█	<i>Z. isselianum</i>
2	<i>Z. isselianum</i>			
3	<i>Z. isselianum</i>			
4	<i>Z. alpestre kupitzense</i>	█	█	<i>Z. kupitzense</i>
5	<i>Z. alpestre kupitzense</i>			
6	<i>Z. isselianum</i>	█	█	<i>Zospeum</i> sp.
7	<i>Z. isselianum</i>			
8	<i>Z. isselianum</i>			
9	<i>Z. cf. amoenum</i>			
10	<i>Z. cf. amoenum</i>	█	█	<i>Z. alpestre</i>
11	<i>Z. isselianum</i>			
12	<i>Z. isselianum</i>			
13	<i>Z. isselianum</i>			
14	<i>Z. alpestre bolei</i>			
15	<i>Z. alpestre bolei</i>			
16	<i>Z. alpestre alpestre</i>			

Figure 4. Overview of morphological assignments, molecular species delimitation results and integrative taxonomic treatment of each of the four clusters, which are indicated by the longitudinal black bars for either the ABDG or the SP approach. Numbers indicate individual specimens; ABGD = Automatic Barcode Gap Discovery; SP = Statistical Parsimony.

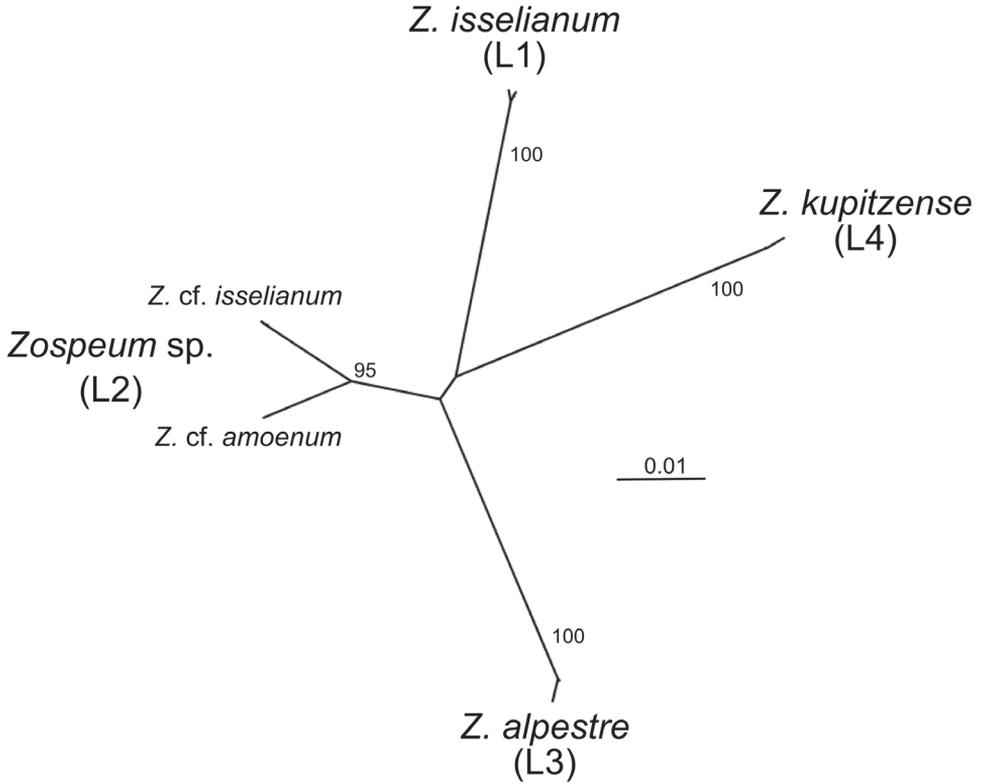


Figure 5. Network analysis for the molecular distinctness of the four delineated *Zospeum* species.

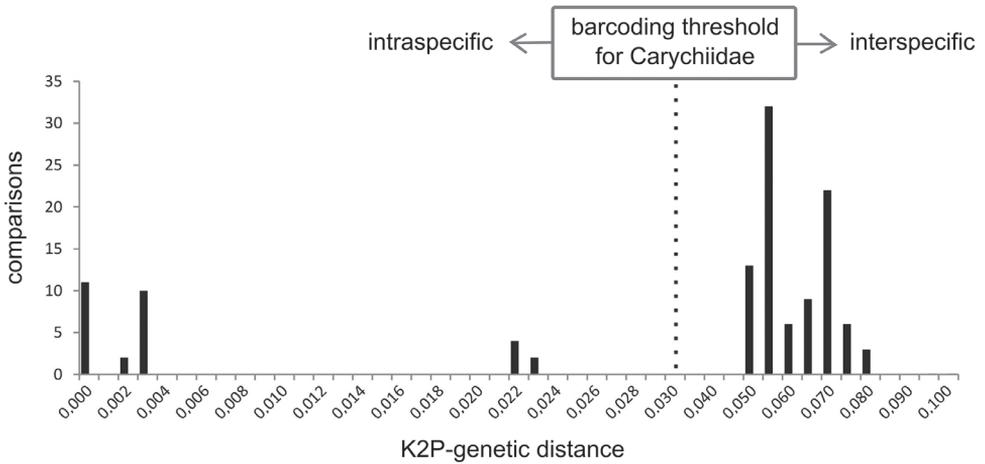


Figure 6. Overview of pairwise genetic distance measurements.

morphospecies		genetic distance table															
		Z. isselianum															
1	Z. isselianum	0.0															
2	Z. isselianum	0.0	0.0														
3	Z. isselianum	0.2	0.3	0.0													
4	<i>Z. alpestre kupitzense</i>	7.2	7.3	7.1	0.0												
5	<i>Z. alpestre kupitzense</i>	7.2	7.2	7.0	0.2	0.0											
6	Z. isselianum	5.5	5.5	5.7	6.2	6.2	Zospeum sp.										
7	Z. isselianum	5.4	5.4	5.6	6.2	6.2	0.0	0.0									
8	Z. isselianum	5.4	5.7	5.7	6.2	6.2	0.0	0.0	0.0								
9	Z. cf. amoenum	5.4	5.4	5.4	7.0	6.8	2.3	2.2	2.2	0.0							
10	Z. cf. amoenum	5.4	5.4	5.4	7.0	6.8	2.3	2.2	2.2	0.0	0.0	Z. alpestre					
11	<i>Z. isselianum</i>	7.0	7.0	7.2	7.6	7.4	5.7	5.6	5.6	5.6	5.6	0.0					
12	<i>Z. isselianum</i>	7.0	7.1	7.1	7.9	7.4	5.8	5.8	5.7	5.4	5.4	0.0	0.0				
13	<i>Z. isselianum</i>	7.0	7.1	7.1	7.9	7.4	5.8	5.8	5.7	5.4	5.4	0.0	0.0	0.0			
14	<i>Z. alpestre bolei</i>	7.2	7.3	7.3	8.1	7.6	6.0	5.9	5.8	5.8	5.8	0.3	0.3	0.3	0.0		
15	<i>Z. alpestre bolei</i>	7.2	7.3	7.3	8.1	7.6	6.0	5.9	5.8	5.8	5.8	0.3	0.3	0.3	0.0	0.0	
16	<i>Z. alpestre alpestre</i>	7.2	7.3	7.3	8.1	7.6	6.0	5.9	5.8	5.8	5.8	0.3	0.3	0.3	0.0	0.0	0.0
specimen		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

Figure 7. Kimura–2-parameter (K2P) genetic distance table in %. Delineated species are marked with boxes. For each individual specimen, its morphospecies designation and genetic distance data to all remaining specimens are given.

Based on the four revealed evolutionary lineages, evidence for delineation using non-molecular methods encompasses four taxonomic consequences:

- 1) *Z. isselianum* Pollonera, 1887 (L1) is defined from the area of its new precise type locality (Turjeva jama, Robič, Kobarid, Slovenia) whereby a neotype is designated and a taxonomic re-description is provided.
- 2) We recognize a highly morphologically variable *Zospeum* sp. (L2) (Konečka zijalka) with conchological affinity to *Z. isselianum* and *Z. cf. amoenum*. However, *Z. amoenum* topotypic material is necessary before species status can be clarified. Histological examination of L2 provides novel insights into the anatomy of *Zospeum* in a comparative framework with other Ellobioidea.
- 3) *Z. alpestre* (L3) encompasses subspecies comprising the *Z. alpestre* complex, including *Z. alpestre alpestre* and *Zospeum alpestre bolei* plus three specimens of *Z. isselianum*.
- 4) *Z. kupitzense* (L4) is elevated from subspecies rank (*Z. alpestre kupitzense*) based on a clear differentiation of columellar configuration and lamellar extension outside the aperture.

Consequence 1: *Zospeum isselianum* (L1) neotype designation including shell morphology and genetics

Taxonomy

Family Carychiidae Jeffreys, 1830

Genus *Zospeum* Bourguignat, 1856

Zospeum isselianum Pollonera, 1887

Figures 2B–F, 8A–F, 9A–F

Zospeum alpestre — Bourguignat 1856 [non *Carychium alpestre* Freyer, 1855]

Zospeum isselianum Pollonera, 1887

Zospeum alpestre — Stossich 1899 [non *Carychium alpestre* Freyer, 1855]

Zospeum alpestre — Giusti and Pezzoli 1982 [non *Carychium alpestre* Freyer, 1855]

Data regarding the original type material. The two syntypes of *Zospeum isselianum* were collected in debris of the Natisone River in the northeastern Italian region of Friuli (Pollonera 1887). Pollonera remarked that A. Tellini collected the debris far north of the town of Cividale and that the specimens could only have originated from the upper, mountainous part of the Natisone valley, namely, the right tributary of the Isonzo River. The Natisone (Nadiža) flows from the Slovenian side of the Julian Prealps and courses the Slovenian-Italian border crossing into Eastern Friuli. Currently, the only known type material consists of a single shell (MZUT M3232) labeled “cotypus” from the Pollonera collection housed in the Museo Regionale di Scienze Naturali Sezione di Zoologia, Torino, Italy (Fig. 2A). Since this specimen (MZUT M3232) is unrecognizable due to Bynesian decay (also called “Bynes disease”), a granular efflorescence of the shell due to a physicochemical reaction involving acetate/formate compound salts and water, which largely erodes the shells (Callomon and Rosenberg 2012, Cavallari et al. 2014), it is no longer taxonomically informative. Moreover, since it cannot maintain nomenclatural stability (ICZN 2014, Art. 75.5), we propose the designation of a neotype (CSR SASA 37013) from the assumed type locality. Material of *Z. isselianum* selected for the neotype here was collected in Turjeva jama, located on the right bank of the Natisone (Nadiža) River by the village of Robič in the municipality of Kobarid, Slovenia. Geographically, this cave is most likely the source of the two shells initially presented to Arturo Issel and later dedicated to him in 1887 by Pollonera.

Original differential diagnosis (Pollonera 1887). “This species just resembles *Z. obesum* Schmidt, *Z. alpestre* Freyer (pars), and *Z. nyctozoilum* Bourg. From the first it differs because it lacks an obsolete columellar fold, because it has the umbilicus more open, the peristome less swollen, and the apex not acute. From the second it differs because it has the umbilicus less cramped, the apex less acute (indeed mammillatus), the sutures deeper and it has the last lap with a lower development. From the third it differs because the right margin is a little arched (not reflected) and it has the umbilicus more open (Issel)” (Pollonera 1887, translated from Italian by Massimo Prodan and Floriana Umani 2014).

Material examined. Neotype (CSR SASA 37013/37013a) (Fig. 8A–E). Conspecifics (BOLD-ID: BARCA121-10, ACCESSION NR: HQ171594) (Weigand et al. 2011)), SMF 341636 (Fig. 2B–F), NMBE 532009 (Fig. 8F): Turjeva jama, Robič, Kobarid, Julian Alps, Slovenia, 46.2435°N, 13.5046°E, alt. ca. 253 m, air temp. in cave 9.3° C, June 1, 2013. Leg. Rajko Slapnik.

Diagnosis. Shell minute, transparent when fresh, conical with entire, half-roundish and more or less thickened peristome; parietal lamella; columella non-introrse, secondary columellar dilatation just above umbilical indentation; columellar lamella inclinate.

Description. Measurements of neotype specimen CSR SASA 37013 (Fig. 8A–E): shell height 1.56 mm, shell diameter 1.07 mm, peristome width 0.75 mm, peristome height 0.84 mm.

Measurements of conspecifics from Turjeva jama are provided in Table 3 (based on neotype and conspecifics of neotype). Shells minute (Table 5), variable in height (1.05–1.63 mm), conical with about 5 whorls, regularly coiled, suture deep, whorls convex, more or less shouldered; aperture semicircular, peristome closely adhering to spire (Fig. 2D), thickened, higher than wide, taking up to ca. 35% of shell height; umbilicus closed, shallow depression; contact between the columellar edge of peristome and last whorl moderately long (0.34–0.59 mm); non-expressed parietal lamella in aperture continues only in traces within shell; parietal plica present or absent (Fig. 8F); penultimate whorl “sinks” into ultimate whorl at upper junction of peristome and penultimate whorl in profile perspective (Fig. 2D); columella twisted but non-introrse, slender, secondary columellar dilatation at base; columellar lamella inclinate, extended (Fig. 8A–D); secondary columellar dilatation equal to ½ distance of maximal upper columellar lamella extension; protoconch with pattern of spiral interconnected pits separated by zones of non-pitted bands (Fig. 9D–E); teleoconch with tightly spaced irregular spiral striae of densely interconnected pits (Fig. 9B, C, F); broader spiral bands interrupt finer rows of pitted striae (Fig. 9B).

Differential diagnosis (Figs 10, 11). Differs greatest from congeners here in columellar apparatus and surface microstructure; differs from *Zospeum* sp. (Konečka zijalka) by the more slender columella, secondary columellar dilatation equal to ½ distance of maximal upper columellar lamella extension, columellar lamella inclinate, extended; *Z. cf. amoenum* (Potočka zijalka) lacks columellar lamella, columella straight cylindrical (Fig. 10J); from *Z. kupitzense* by the planar less inclinate orientation of columellar lamella (Fig. 10A–F), less pronounced parietal lamella than in *Z. kupitzense* (Ložekarjeva zijalka) (Fig. 10E); from *Z. alpestre* (Kamniška jama) by the more slender columella, secondary columellar dilatation at base, columellar base of *Z. alpestre* clavate inflated, moderately attenuate (Fig. 10G); irregular striation of tightly interconnected pits of upper teleoconch differs from regular impressed wavy bands on teleoconch of *Z. kupitzense* (Fig. 9I–L); differs from rather regularly spaced rows of interconnected pits on teleoconch of *Z. amoenum* (Ihanščica cave). The differential diagnosis of genetic data is presented with the respective BOLD-ID numbers in Table 2.

Distribution. Although we question past distribution records (Fig. 1, Table 1), *Z. isselianum* likely occurs within a narrow radius encompassing the Southeastern Alpine

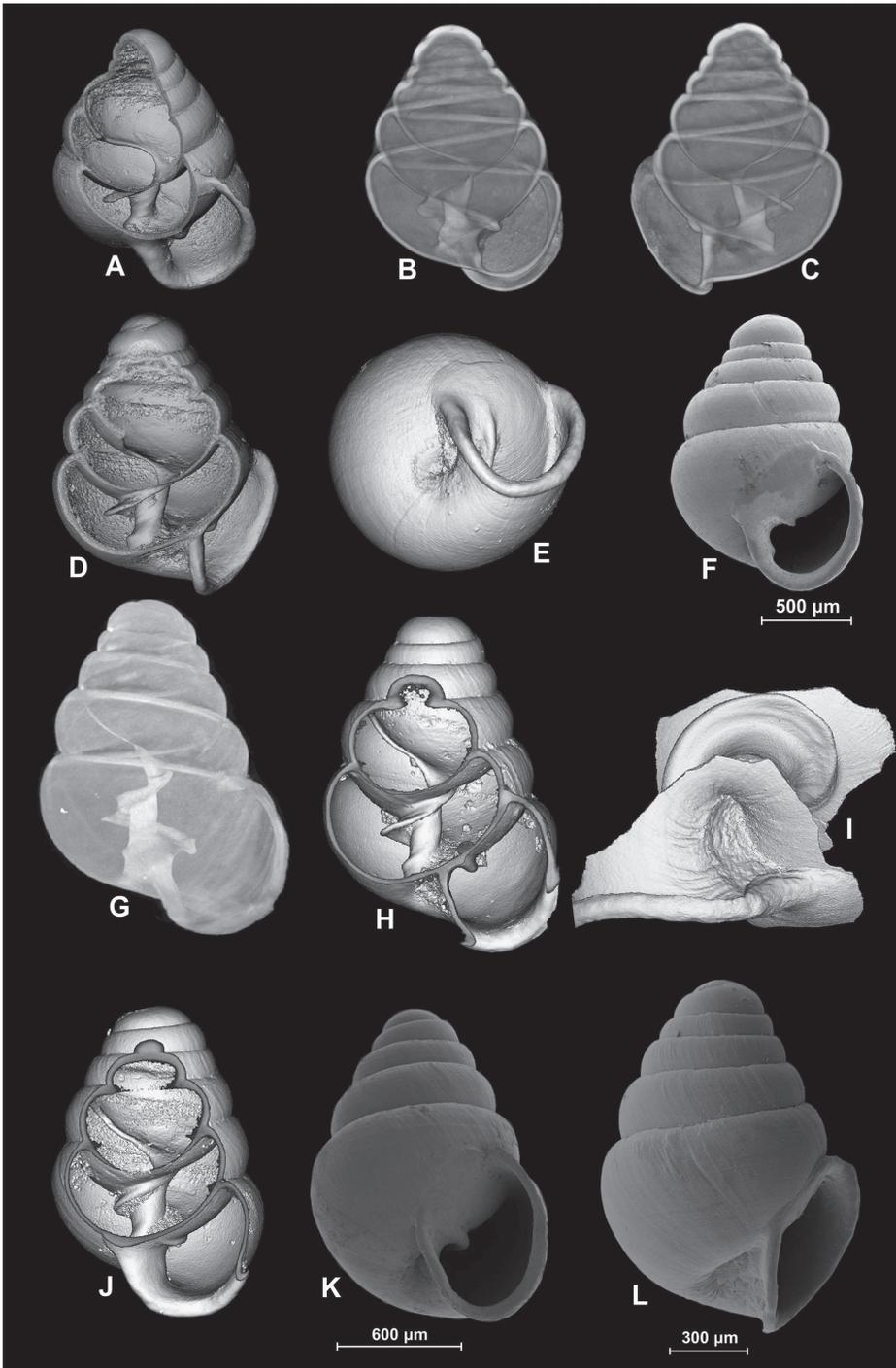


Figure 8. NanoCT-SEM Plate 2. **A-F** *Zospeum isselianum*, neotype (CSR SASA 37013), Turjeva jama **G-H** *Zospeum isselianum* (SMNH 2216), Jama na Zgornjih Brsnikih **I-L** *Zospeum* sp. (CSR SASA 37698a), Konečka zijalka.

Table 3. *Zospeum isselianum* conspecifics from neotype locality (Turjeva jama), CSR SASA 37013/01–10, Turjeva jama, Robič, Kobarid, Slovenia. **SH** Shell height. **SW** Shell width. **PH** Peristome height. **PD** Peristome diameter. **Lcol** Contact between the columellar edge of peristome and last whorl, all in mm.

	SH	SW	PH	PD	Lcol	SW/SH	AW/AH	Lcol/SH
37013/01	1.05	0.91	0.70	0.47	0.34	0.865	0.676	0.321
37013/02	1.35	1.02	0.71	0.58	0.46	0.755	0.813	0.341
37013/03	1.44	0.96	0.76	0.62	0.49	0.670	0.811	0.344
37013/04	1.36	0.95	0.69	0.53	0.54	0.698	0.766	0.396
37013/05	1.30	0.97	0.77	0.60	0.58	0.744	0.775	0.444
37013/06	1.39	0.95	0.70	0.58	0.52	0.686	0.825	0.377
37013/07	1.36	0.98	0.68	0.57	0.44	0.721	0.842	0.323
37013/08	1.37	0.92	0.68	0.60	0.54	0.673	0.893	0.396
37013/09	1.41	1.02	0.72	0.61	0.53	0.726	0.847	0.375
37013/10	1.31	0.96	0.68	0.56	0.55	0.737	0.813	0.423

Table 4. Shell dimensions. Mean, maximum (max), minimum (min), and standard deviation (sd) of shell measurements of 3 populations of *Zospeum isselianum*, Turjeva jama, N=40; Jama na Zgornjih Brsnikih N=40 and *Zospeum* sp. Konečka zijalka, N=32. **SH** Shell height. **SW** Shell width. **PH** Peristome height. **PD** Peristome diameter. **Lcol** Contact between columellar edge of peristome and last whorl, all in mm.

	SH	SW	PH	PD	Lcol	SW/SH	PH/PD	Lcol/SH
<i>Zospeum isselianum</i> Turjeva jama								
mean	1.35	0.97	0.70	0.58	0.49	0.72	0.83	0.36
max	1.63	1.09	0.84	0.62	0.59	0.86	0.92	0.45
min	1.05	0.91	0.65	0.47	0.34	0.63	0.68	0.24
sd	0.086	0.036	0.033	0.028	0.052	0.039	0.046	0.038
<i>Zospeum isselianum</i> Jama na Zgornjih Brsnikih								
mean	1.53	1.07	0.75	0.60	0.41	0.70	0.80	0.27
max	1.63	1.12	0.93	0.87	0.58	0.76	0.93	0.39
min	1.38	0.87	0.67	0.54	0.29	0.55	0.68	0.18
sd	0.051	0.055	0.044	0.053	0.060	0.037	0.048	0.0417
<i>Zospeum isselianum</i> Konečka zijalka								
mean	1.37	1.02	0.69	0.61	0.53	0.75	0.87	0.35
max	1.60	1.25	0.80	0.74	0.67	0.84	1.06	0.40
min	1.19	0.93	0.58	0.51	0.42	0.68	0.76	0.30
sd	0.084	0.066	0.051	0.039	0.066	0.037	0.055	0.053

region of contiguous cave and river systems of northeastern Italy, southern Austria, Slovenia and northwest Croatia (Bole 1974, Maier and Bole 1975, Mildner 1976, Slapnik 1991, 1994, Slapnik and Ozimec 2004).

Ecology. Live *Z. isselianum* were found in Turjeva jama on mud and muddy walls at the end of the cave and adjacent to the central pit located in the middle of the cave. Bats were seen in the vicinity of the collection site.

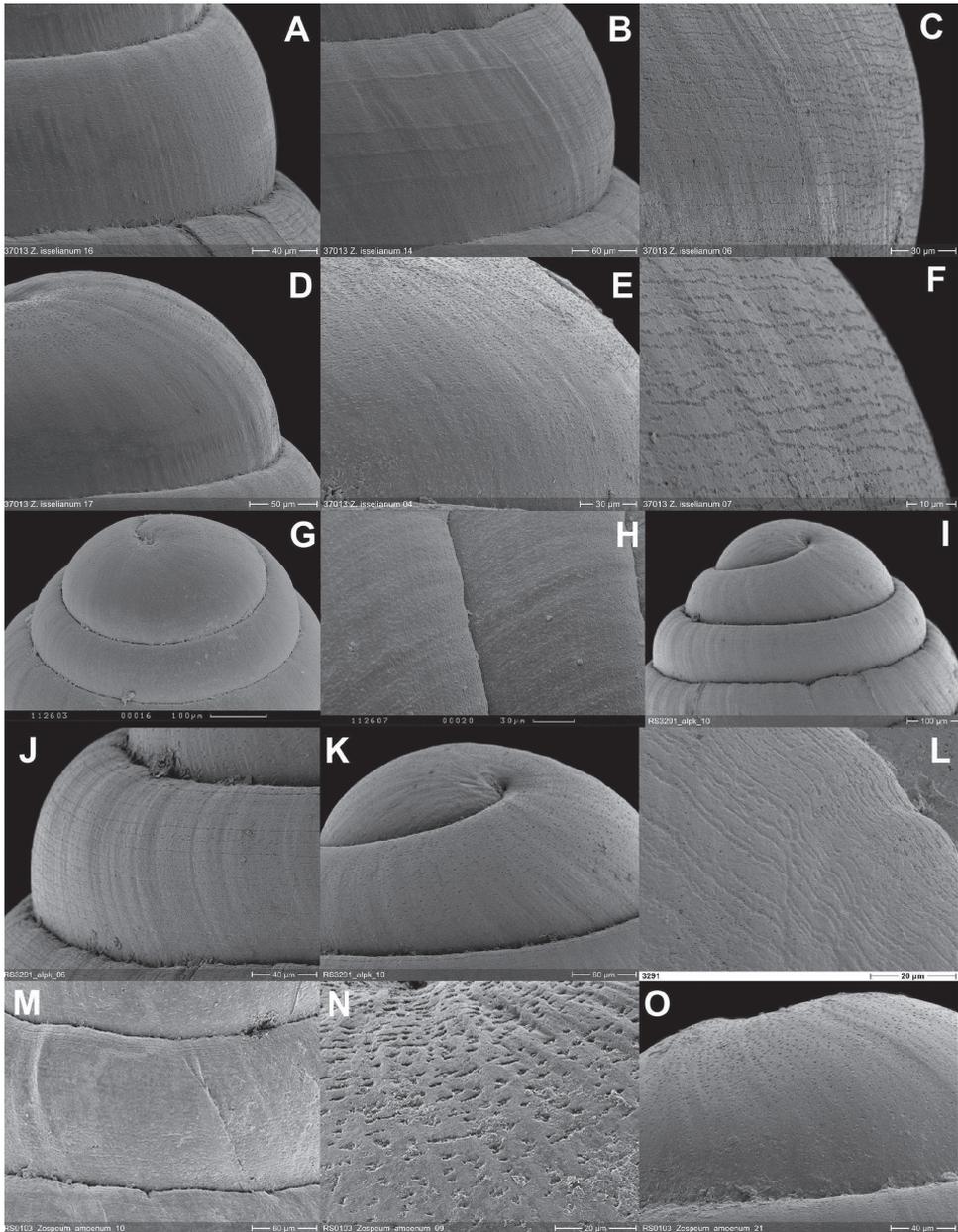


Figure 9. SEM Microstructures Plate 3. **A–F** *Zospeum isselianum* (CSR SASA 37013 conspecific), Turjeva jama **G–H** *Zospeum* sp. (CSR SASA 21675), Konečka zijalka **I–L** *Zospeum kupitzense* (SMNH 3291), Ložekarjeva jama **M–O** *Zospeum amoenum* (RS103), Ihanščica cave.

Conservation. In the caves sampled for this study, empty shells were sparsely found in sediment and live individuals sparsely populated certain cave walls during the summer season. These findings suggest that *Z. isselianum* occurred there for more than

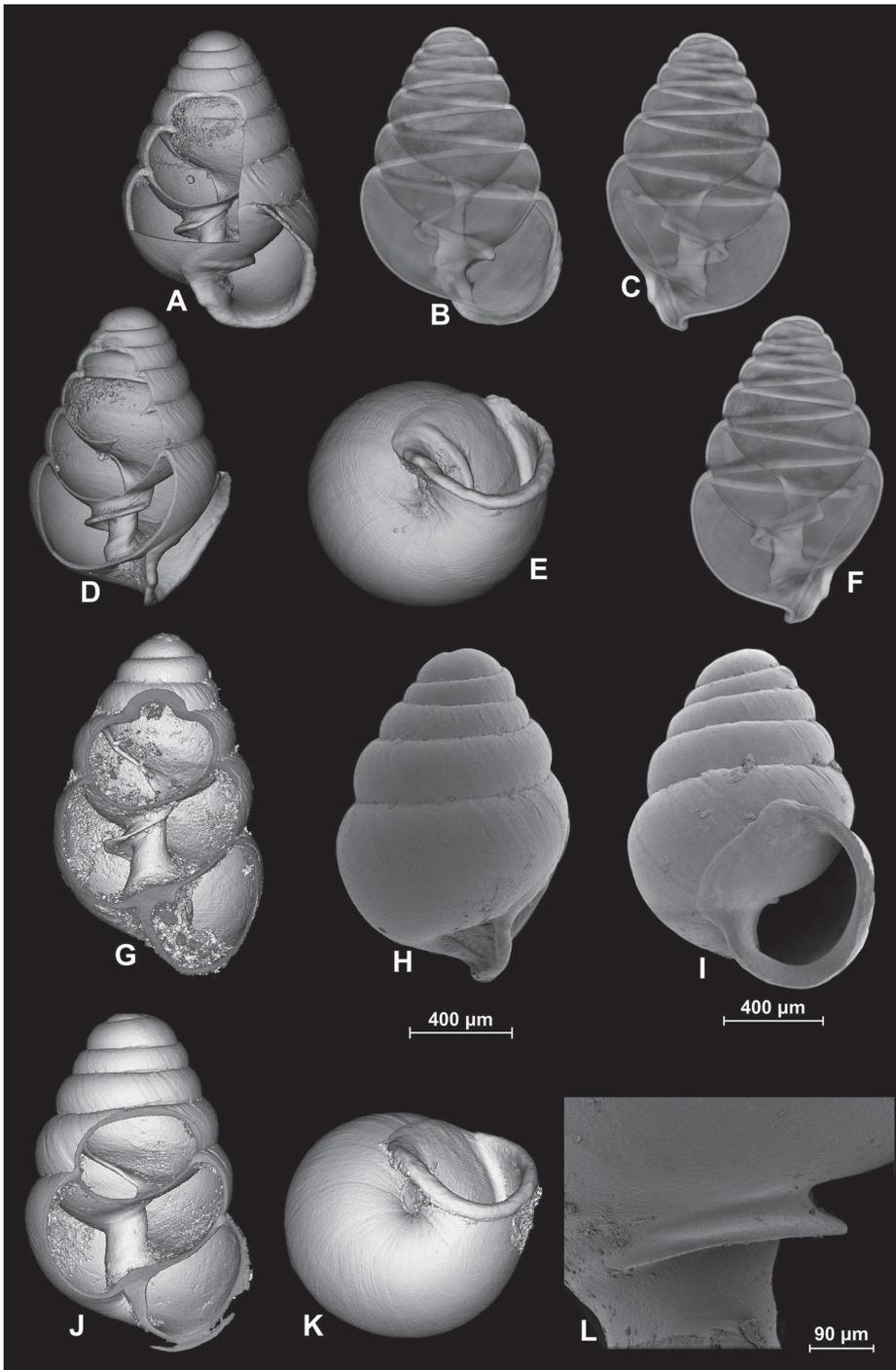


Figure 10. NanoCT-SEM Plate 4. **A-F** *Zospeum kupitzense* (RS3291), Ložekarjeva zijalka **G.** *Zospeum alpestre* (SMNH 2216), Kamniška jama **H, I-L** *Zospeum iselianum* Ihanščica cave **J-K** *Zospeum amoenum* (RS59), Potočka zijalka.

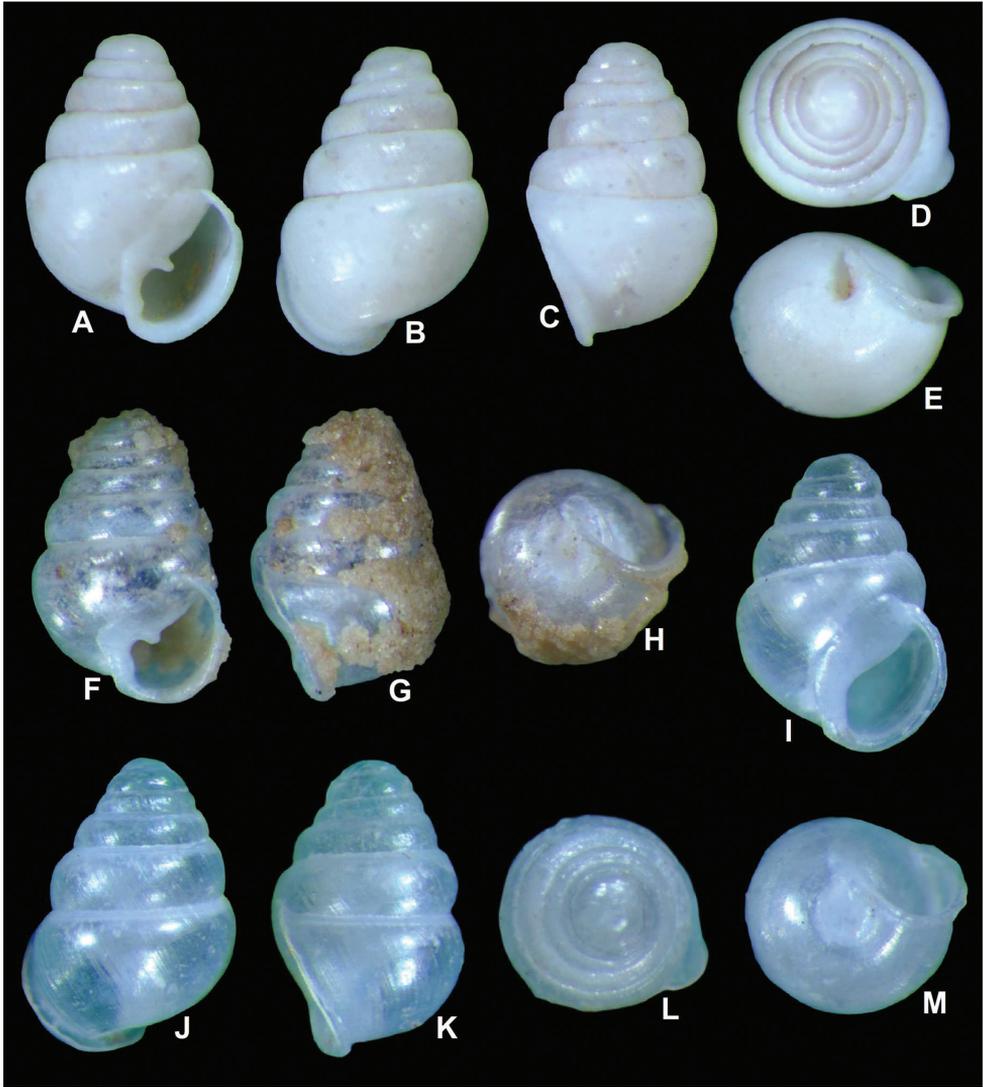


Figure 11. Type and shell images Plate 5: **A–E** *Zospeum kupitzense* holotype: (SMF 256354) **F–H** *Zospeum kupitzense* (SMNH 3291), Ložekarjeva zijalka **I–M** *Zospeum amoenum* (RS59), Potočka zijalka.

one season, and that these populations are/were not immediately threatened. Still, on a global scale, its distribution is fragmented and likely limited to far less than 312 caves within a radius of 20,000 km². In conjunction with the categories for the IUCN Red List (IUCN Standards and Petitions Subcommittee 2014), it can be considered a taxon of Least Concern (LC) since *Z. isselianum* is (potentially) known from many sites and these populations seem to be rather stable. Habitat disturbance through human agency poses the greatest threat.

Remarks. This species shows a wide range of variability in the shell. The parietal lamella with its nondescript shape and configuration has been considered characteristic

(Bole 1974, Slapnik 1991). The size and shape of the shell and aperture in conjunction with the visible parietal lamella indicate the very close relationship with *Z. alpestre* (see Bole 1974) and with *Z. amoenum* (see Slapnik 1991) in eastern Slovenia. The columella differs from congeners in this study by the presence of a secondary columellar dilatation at the base and an inclinate, extended columellar lamella. Superficial microstructure on the protoconch consists of spirally arranged pits interspersed by medium to wide zones of smooth non-pitted bands. The teleoconch shows a deep suture and irregular rows of tightly interconnected pits. Sometimes microscopic white chalky bands above and below the suture can be seen.

Consequence 2: Histological study of *Zospeum* sp. (Konečka zijalka) ultrastructure

Digestive system

As known for pulmonates, the anterior digestive system (Fig. 12) of *Zospeum* sp. (Konečka zijalka) has a muscular buccal mass containing the radular sac (rs) and a radula (r) bearing many teeth. The mouth is a slit located between the oral lappets. A crescent-shaped chitinous jaw (j) lies in the anterior part of the buccal mass (Fig. 12A). The jaw serves to press against and help grip the substratum, supporting interactions of the buccal mass during feeding (Morton 1955). In sync with the ellobioid scheme, the oesophagus (oe) is simplified (Barker 2001), bearing a thick cuticular sheath (cs) and extends straight into the unbranched section of the visceral cavity, where it widens into a thin-walled foregut. The anterior part of the visceral tract includes the oesophagus and foregut (fg), with associated, paired salivary glands (foregut glands) (sg) with ducts discharging into the posterior section of the buccal chamber (pharynx). The kidney (k) here is proportionately very large in respect to the overall size of this tiny snail. In our serial section (Fig. 12A) and in Dörge's (2010, unpublished data) partial 3D reconstruction (Fig. 15), the kidney encompasses ca. 14% of the entire volume of the cephalopedal mass. The heart complex (h) consisting of a single auricle and a single ventricle abuts the kidney at the bottom of the kidney sac. The foregut leads to the median section of the stomach, which is contained in a highly muscular girdle to form a contractile gizzard (g) (Fig. 16). A thick layer of cuticle (cu) lines the stomach. The muscular gizzard triturates the intesta (il), which in *Zospeum* sp. (Konečka zijalka) here, is composed of much mineral matter likely enriched with microorganisms and fungal tissue. The intesta of the stomach shows alkaline character inferable by the intense turquoise-coloured flecks of the toluidine stain. The elaborate muscular plates (mp) of the *Zospeum* sp. (Konečka zijalka) gizzard and the prevalent cuticle throughout the whole lumen (Fig. 16B) underscore the ellobioid tendency towards structural deviation of the gizzard as a grinding organ from the rest of the stomach (Barker 2001). The gizzard in *Zospeum* sp. (Konečka zijalka) here is overall much more elaborate than that of *Z. speleum schmidtii* (Maier 1982, unpublished data). The thick epithelial wall of the stomach most in contact with the intesta shows columnar (cc) digestive cells

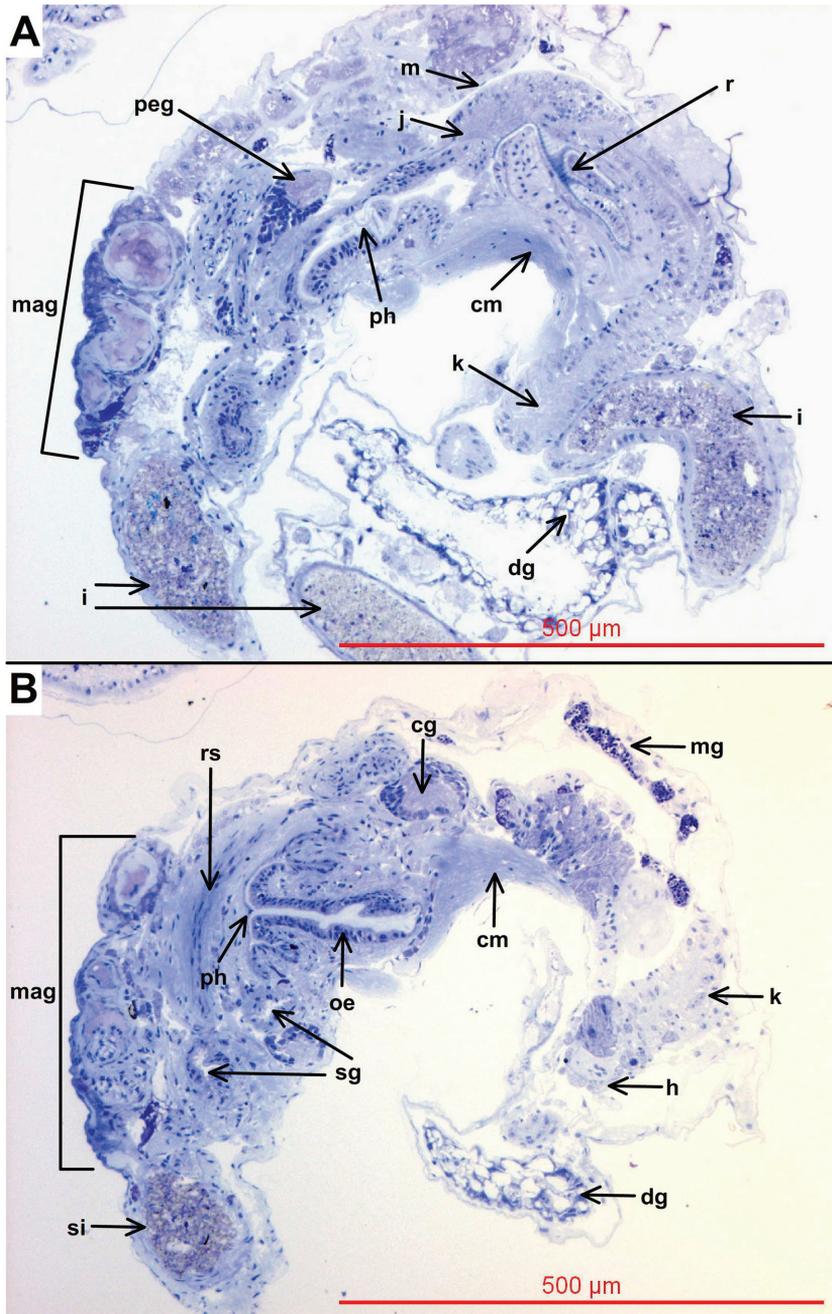


Figure 12. Light micrograph showing histological overview of the digestive system of *Zospeum* sp. (Konečka zijalka) (CSR SASA 21675). **A** Mouth and buccal apparatus. Mouth slit between oral lappets (m), crescent-shaped jaw (j), radula (r), pharynx (ph), digestive gland (dg), intestine (i), kidney (k), columellar muscle (cm), and pedal ganglia (peg) **B** Mantle gland (mag), sigmoid intestine (si), radular sac (rs), cerebral ganglion (cg), mucus glands (mg), kidney (k), heart showing auricle and ventricle (h), digestive gland (dg), columellar muscle (cm), oesophagus (oe) and salivary glands (sg).

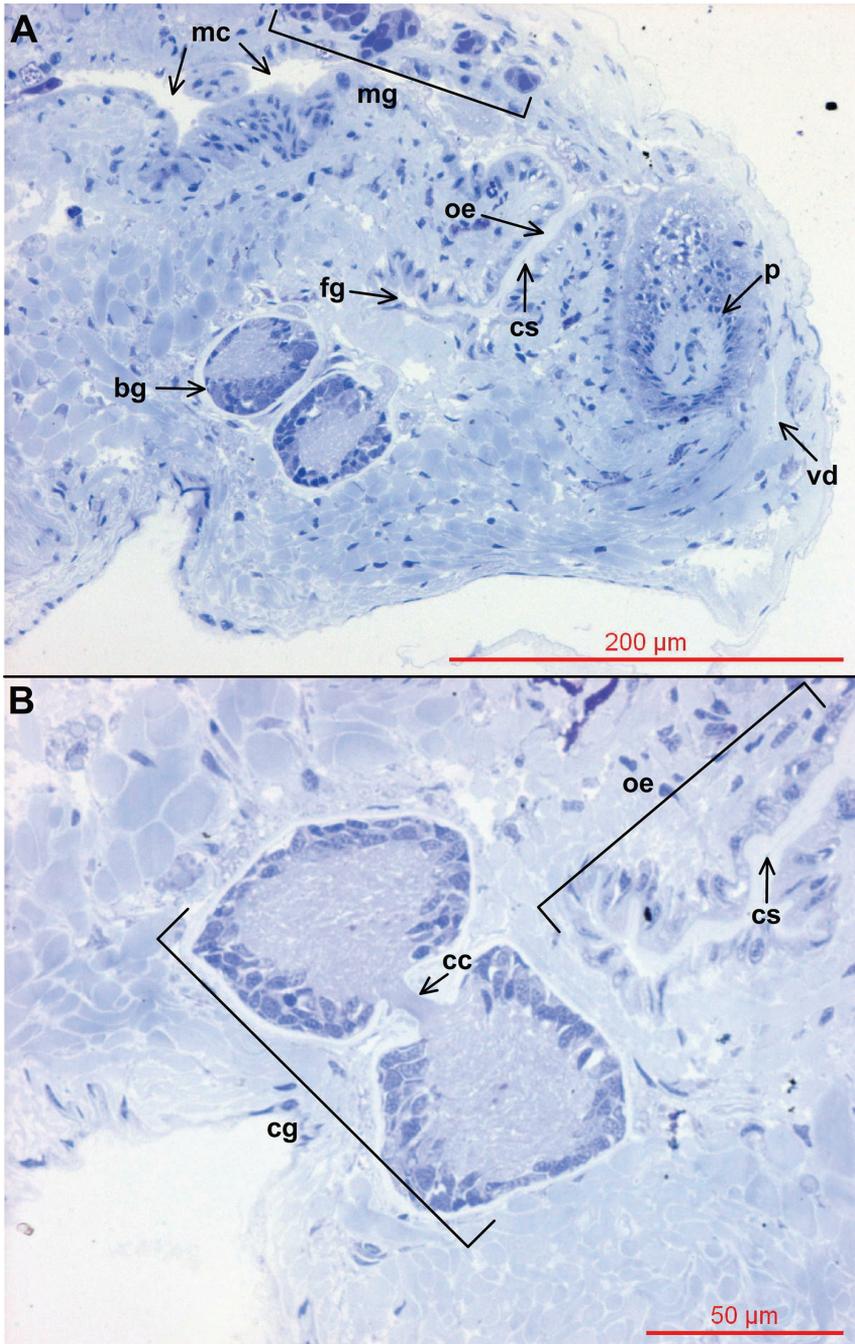


Figure 13. A Light micrograph showing the histological appearance of *Zospeum* sp. (Konečka zijalka) (CSR SASA 21675) with the mantle cavity (mc), mucus glands (mg), oesophagus (oe) bearing thick cuticular sheath (cs) and leading into the crop (c), the buccal ganglia (bg), and the cephalopodial portion of the reproductive system including penis (p) and vas deferens (vd) **B** Paired cerebral ganglia united by the cerebral commissure situated just dorsal to the origin of the oesophagus in the buccal mass.

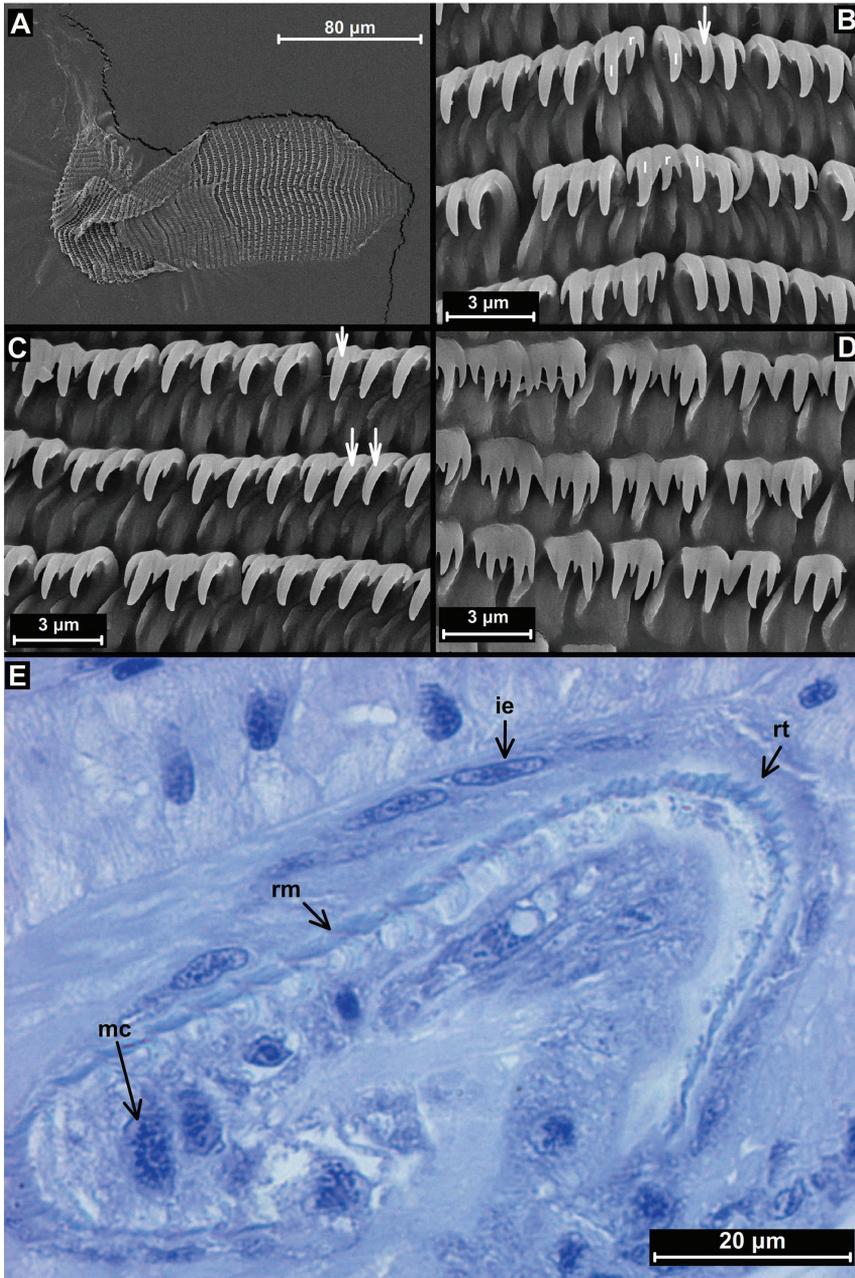


Figure 14. Images of the radula of *Zospeum isselianum* (Turjeva jama) (CSR SASA 37013 conspecific) and *Zospeum* sp. (Konečka zijalka) (CSR SASA 21675). **A–D** SEM of the radular ribbon of topotypic *Z. isselianum* **A** Radular ribbon showing adhesive layer of contact zone with odontophore **B** SEM overview showing rachidian teeth (r) and lateral teeth (l) **C** Rows of lateral teeth bearing fine medial groove **D** Marginal teeth **E** Light micrograph showing the histological appearance of the radular sheath of *Zospeum* sp. (Konečka zijalka) showing the inferior epithelium (ie) attached to the radular membrane (rm) mineralizing cells (mc) with degenerating nuclei and radular teeth (rt).

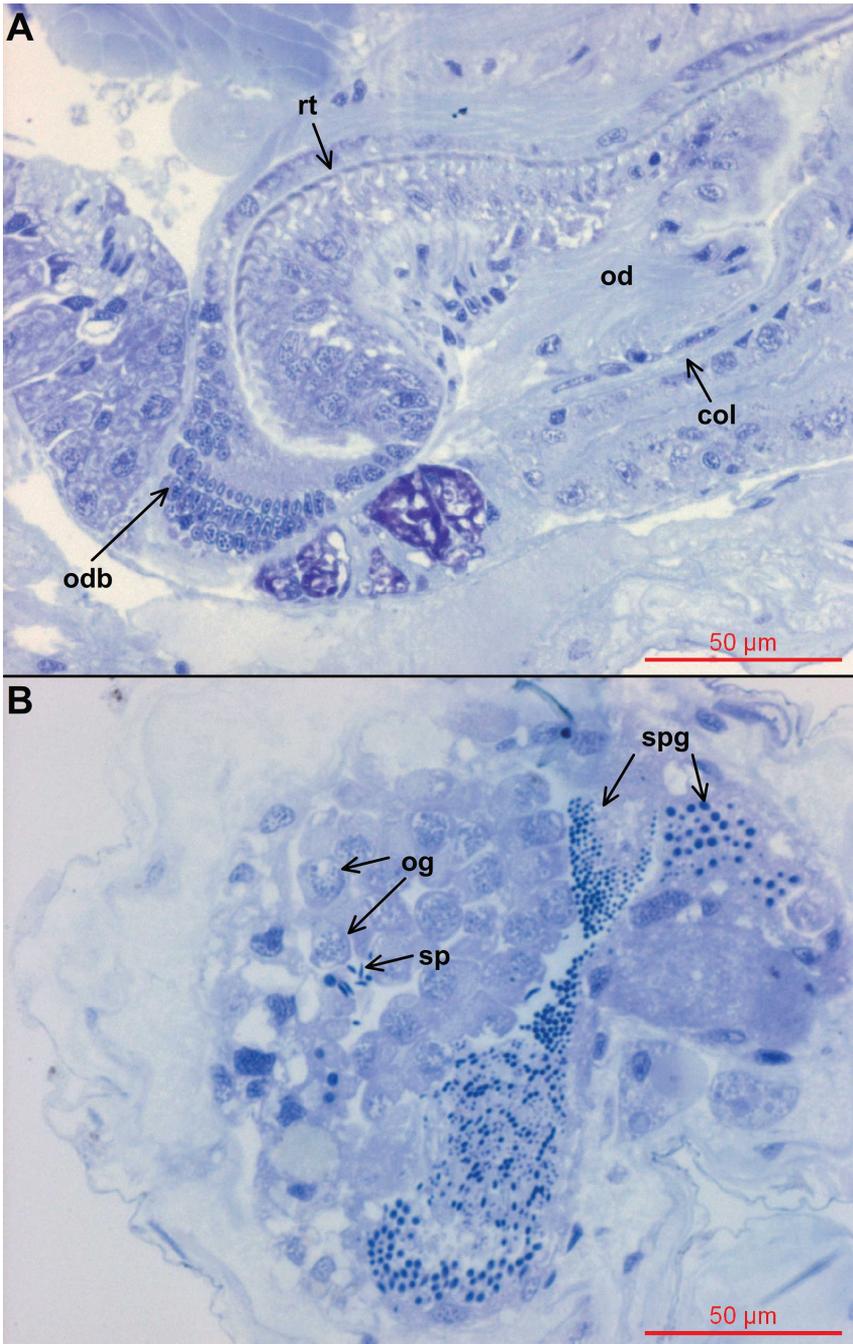


Figure 15. Light micrograph showing histological appearance of the radular complex of *Zospeum* sp. (Konečka zijalka) (CSR SASA 21675). **A** Odontoblasts (odb) grouped in lower posterior section of radular sheath, radular teeth (rt), odontophore (od), and collostyle (col) **B** Section through an acinus of the ovotestis showing some stages of development of sustentacular cells (Sertoli cells) (sc) with spermatogonia (spg), spermatids (sp) and oogonia (og).

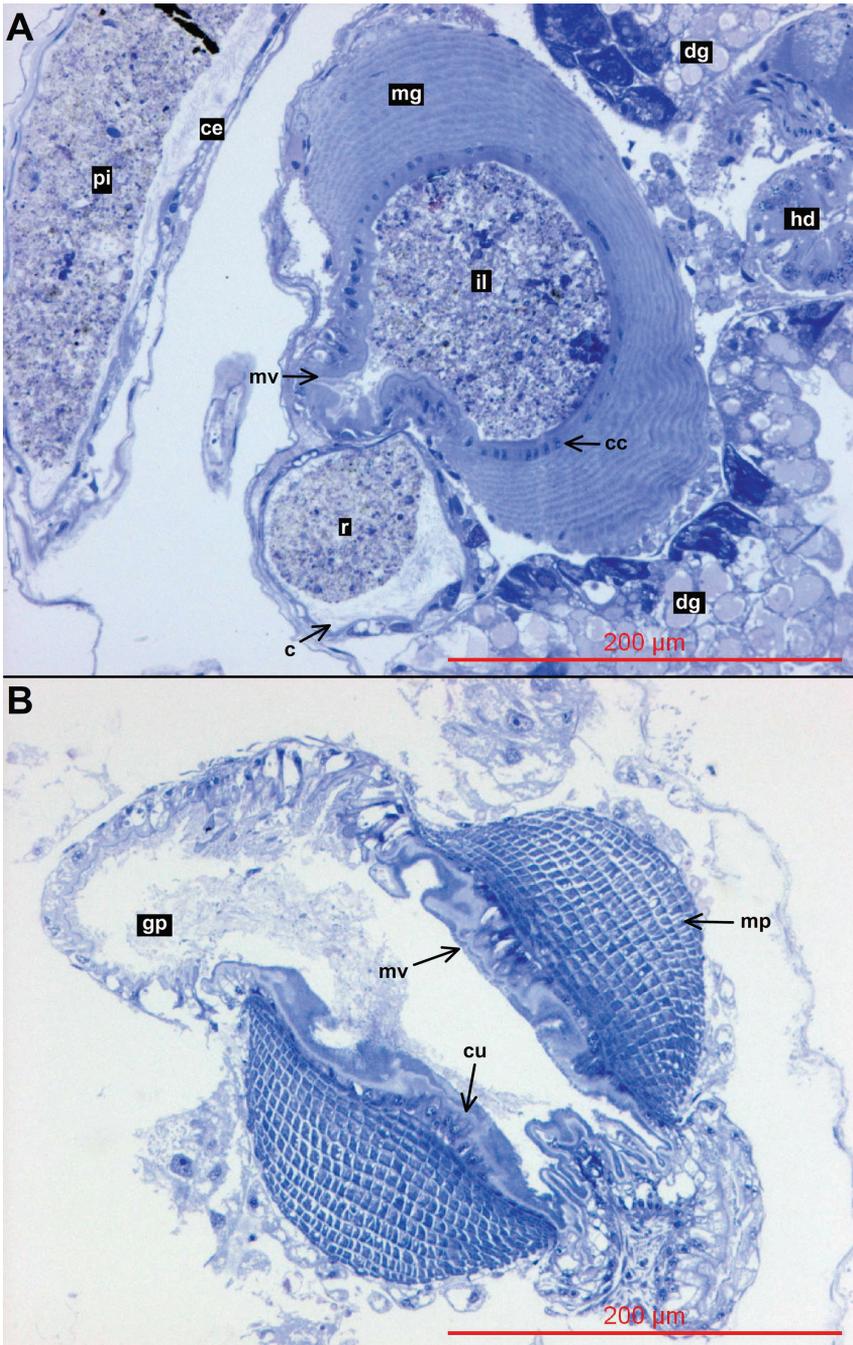


Figure 16. Light micrograph of the histological appearance of the digestive tract of *Zospeum* sp. (Konečka zijalka) (CSR SASA 21675). **A–B** Bilobed muscular gizzard (mg) with epithelium showing columnar cells (cc) with microvillous apical surfaces (mv), posterior loop of the intestine (pi) with ciliated epithelium (ce) **B** Elaborate muscular plates (mp), thick cuticle (cu), highly ciliated rectum (r), gastric pouch (gp), digestive gland (dg) and hermaphroditic duct.

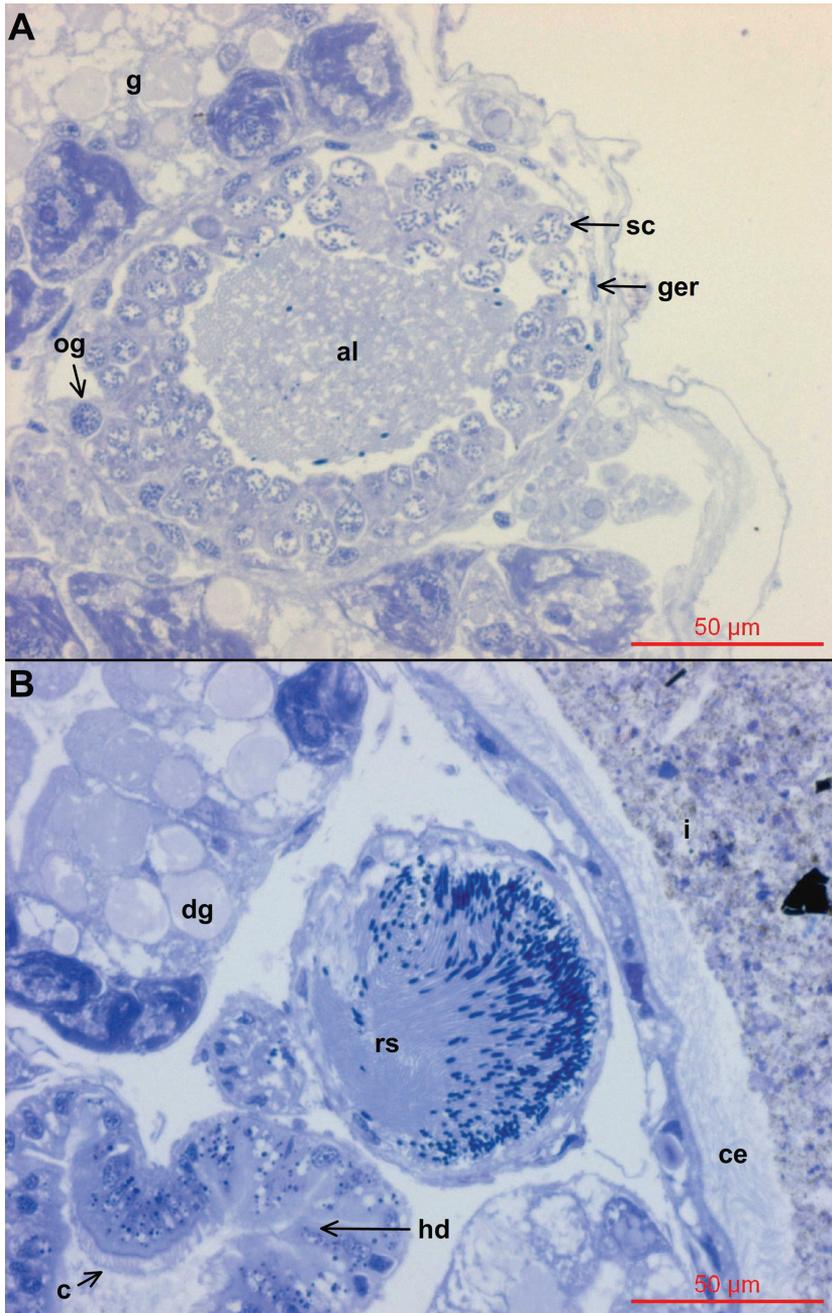


Figure 17. Light micrograph of a section of the reproductive tract of *Zospeum* sp. (Konečka zijalka) (CSR SASA 21675). **A** Gonad (g) with acinus of the ovotestis including the germinal epithelial ring (ger) surrounding the acinar lumina (al), sustentacular cells (Sertoli cells) (sc) and oogonia (og) **B** Digestive gland (dg), hermaphroditic duct (hd) lined with cilia (c), posterior loop of the intestine (pi), receptaculum seminis (rs) containing allospermatozoa with spermatozoa oriented towards and apical ends touching the epithelial cells, and long motile cilia (ce) of intestine (i).

covered on the apical surface with microvilli (mv). The paired lobes of the glandular, voluminous digestive gland (dg) (Figs 12, 16, 17) are considered by Sumner (1965) to take on multiple functions in nutrient absorption, processing, secretion and excretion in pulmonate snails. In conjunction with the mantle gland (mg), the digestive gland is considered to provide materials for the organic crystalline matrix deposited in shell regeneration (Albolins-Krogis 1968).

The long intestine of *Zospeum* is visible from the outside of the shell when the snail is alive. The intestine encompasses an anteriorly directed loop frequently seen amidst other members of the Ellobioidea (Barker 2001) (Fig. 16). The posterior loop (pi) is large in surface area with an abundance of motile cilia (Fig. 16A). A remarkable degree of ciliation is also seen in the rectum. Long fecal strings are characteristic of the Carychiidae as well as for the Ellobioidea in general (Barker 2001).

Though not part of the digestive system but nonetheless encompassed in the broader scope of these slides, the mantle gland (mg) (Fig. 12) shows two different types of cellular composition, which more or less blend into each other within a circular, uniform layer of cuticle. This compact group of cellular packets is smaller and less extensive than that reported for *Z. spelaeum schmidtii* (Maier 1982, unpublished data).

Radula

The Carychiidae are considered to possess the simplest ellobioid radula (Martins 2007), bearing the typical symmetrical rachidian tooth accompanied by a series of lateral teeth and about the same number of tightly aligned marginal teeth. The radula of *Z. isselianum* (Turjeva jama, neotype locality) shows the tricusperate “*Carychium*-type” radula defined by Martins (2007) and reported by Jochum (2011, fig. 3) for *Zospeum* collected in Konečka zijalka. The cusps of the lateral and marginal teeth are flanked by an apposition of an additional denticle. The radular ribbon of topotypic *Z. isselianum* (Turjeva jama) shows the adhesive layer designating the zone of tensile strain and contact with the muscular odontophore (od) (Fig. 14A). In the histological section of *Zospeum* sp. (Konečka zijalka) (Fig. 15A), this subradular membrane is comprised of cuticula. It is formed at the opening of the radular sheath. The rachidian tooth (r) of *Z. isselianum* (Turjeva jama) is rather small (Fig. 14B). The mesocone is long while the ectocones are shorter and about one half the length of the mesocone. The tricusperate lateral teeth (l) are larger, bearing a slightly curved, uniformly long mesocone flanked by an endocone and an ectocone of differing lengths. The marginal teeth proceed from the laterals in cusp number from 3 to 4 to 5, whereby the third and fifth cusp of each tooth are reduced in length and the second and fourth cusps are about the same length (Fig. 14D). In general, our observations match those of Giusti (1975, pl. 1) for *Z. tellenii* (= *Z. spelaeum costatum* (Freyer, 1855)) with the exception of a fine median groove midway down the mesocone in some of the lateral teeth (Fig. 14B–C). So far, this groove has only been observed on the more rounded, perhaps older teeth of the radulae from *Zospeum* sp. from Konečka zijalka (Jochum

2011, Dörge 2010, unpublished data) and from topotypic *Z. isselianum* here from Turjeva jama. As is known for pulmonates, the chitinized radular teeth are uniformly shaped in longitudinal columns, but vary markedly in the transverse direction on the radular membrane (Luchtel et al. 1997). The radular sheath is comprised of the radula membrane (rm), which is closely attached to the inferior epithelium (ie). Mineralizing cells (mc) can be seen in close contact with the developing teeth (Luchtel et al. 1997) (Fig. 14E).

Each column of teeth is formed by a single set of a number of odontoblasts (odb) located at the lower posterior end of the radular sheath as seen here for *Zospeum* sp. (Konečka zijalka) (Fig. 15A) (Mackenstedt and Märkel 1987). The shape, size and position of radular teeth are determined by the position, size and secretion rate of the odontoblasts (Mischor and Märkel 1984). These cells produce the matrix of the radula, whereas the superior and inferior epithelia (ie) (Fig. 14E) serve towards the maturation and transport of the *Zospeum* radula here (Mackenstedt and Märkel 1987). The colostyle (col) (Fig. 15A) is considered to play a role in supplying nutrients to the epithelia of the radular sac and may be integral in the disposal of degenerated cells (Mackenstedt and Märkel 2001).

Reproductive structures

One of the most prominent aspects of the cephalopedal region of *Zospeum* sp. (Konečka zijalka) here is the retracted conical penis and the long vas deferens embedded in the body wall, running parallel to but independent of the penis (Fig. 13A). The penial sheath in our cross section is sheer with this copulatory structure. The vas deferens follows a straight course before taking an abrupt left turn down the lower right hand side of the organ to meet the insertion point of the penis. This could possibly be due to contraction of the animal during preservation and not necessarily reflect the situation when the animal is alive.

The orange-yellowish gonad (when fresh) is situated between the uppermost lobes of the digestive gland in the hollow, apical whorls of the teleoconch (Fig. 8A–D). The lobes of the gonad comprise densely packed acini containing germinal cells (oogonia (og) and spermatogonia (sp)) and somatic Sertoli cells (supporting cells) (sc) and follicular cells (Fig. 17A). The male germ cells appear to develop and mature earlier in *Zospeum* sp. (Konečka zijalka) than the female germ cells (Fig. 15B). Diverse studies in other pulmonates have shown that oogenesis occurs in the acinar cortex and spermatogenesis in the compact, medullar region within the germinal ring (ger) situated in the neck of each acinus (Gómez 2001) (Fig. 17A). The acinar ducts of the ovotestis coalesce to form the hermaphroditic duct, whose projecting folds are lined by a dense layer of ciliated epithelium (Fig. 17B). The receptaculum seminis (rs) contains allospermatozoa, whose apical ends are oriented towards the epithelial wall (Fig. 17B).

Central nervous system (CNS)

The CNS of *Zospeum* sp. (Konečka zijalka) follows that of Maier (1982, unpublished data) described for *Z. spelaeum schmidtii* and Morton (1955), Martins (1996) and Harry (1998) for *Carychium*. Our observations conform, in part, to the general ellobioid plan of the central nervous system consisting of five pairs of ganglia (cerebral, pleural, pedal, parietal and buccal). However, since the position of the animal in the individual histological sections was constricted, the parietal and pleural ganglia could not be seen in this specimen. Martins (1996) described three types of CNS of which the Ellobiinian-carychiinian type (figs 24.1H–L) consists of a wide visceral ring with a very short parieto-visceral connective. In *Zospeum* sp. (Konečka zijalka), the two cerebral ganglia (cg) (Fig. 13B) are connected by an arching cerebral commissure (cc) over the oesophagus. In the immediate vicinity of the pharynx are the buccal ganglia (Fig. 13A). The pedal ganglion is particularly prominent due to its larger elongated form adjacent to the pharynx (Fig. 12A).

Consequence 3: Relegation of *Z. alpestre bolei* into synonymy of *Z. alpestre* with differential diagnosis of L3, including context to L2

Character analysis of the shell of allied species

Z. isselianum (RS0103) from Ihanščica cave (Figs 10H, I, L, 11I–M) though presented here in different perspectives, differs by the presence of a columellar lamella from the conchologically identified (RS) *Zospeum amoenum* from Potočka zijalka without a columellar lamella. This individual, though conchologically identified as *Z. amoenum* in a previous era, fits rather the morphology for *Zospeum* sp. as viewed from the backside of the ultimate whorl (Fig. 10H, I, L). Application of nano-CT enables a clear identification based on lamellar and columellar configuration of this shell. *Zospeum amoenum* from Potočka zijalka (Fig. 10J–K) on the other hand, has a smooth, non-elaborated cylindrical columella lacking a columellar lamella. In sync with morphological work by Bole (1974, fig. 3), the shell from Potočka zijalka is conchologically *Z. amoenum*.

The robust, broadly clavate columella of *Zospeum alpestre* of Kamniška jama (Fig. 10G) and the straight unelaborated cylindrical form of *Zospeum amoenum* from Potočka zijalka differ significantly from the slender, twisted forms of *Z. isselianum* from Turjeva jama (Fig. 8A–D) and *Z. isselianum* from Jama na Zgornjih Brsniških (Fig. 8G–H). It appears that the smooth, robust columella in conjunction with the reduced, unelaborated columellar lamella of *Zospeum* sp. from Konečka zijalka (Fig. 8J) presents a unique, yet almost intermediary form to those of *Z. alpestre* (Kamniška jama) (Fig. 10G), *Z. amoenum* (Potočka zijalka) (Fig. 10J) and *Zospeum kupitzense* (Fig. 10A–F). The columellar lamella of *Z. alpestre* (Fig. 10G) on the other hand, is

thin and finely sinuous, extending to a brief horizontal plane within the penultimate whorl (Fig. 10G). The columellar lamella of *Zospeum* sp. (Konečka zijalka) (Fig. 8J) is robust and thick in contrast to the almost filigree form of the former. The lamella does not form a shelf-like projection but continues its slight, sinuous turn rather tightly about the columella, ending as a strong parietal element in the aperture.

Consequence 4: Differential diagnosis for *Z. kupitzense* (L4)

Zospeum kupitzense (Figs 9I–L, 10A–F, 11A–H) shows yet distinct and different shell morphology. Interconnecting shallow pits tend already on the protoconch to form differentiated concentric rows (Fig. 9–L). These rows develop into regularly spaced, incised orderly bands on the second whorl (Fig. 9J). By the third whorl, they become more densely spaced and irregularly wavy (Fig. 9L). Internally, *Z. kupitzense* shows a rather slender but more truncated columella than in *Z. isselianum* (Turjeva jama) (Fig. 8A–D) and *Z. isselianum* from Jama na Zgornjih Brsnikih (Fig. 8G–H). However, like the two *Z. isselianum* forms, *Z. kupitzense* shows a secondary columellar dilatation at the base of the columella, which forms the shallow, but more obliquely positioned umbilical notch. The columellar lamella (Fig. 10A–D, F) maintains a similar, slight sinuous extension in sync with the contour of that in *Z. alpestre* (Fig. 10G). However, the columellar lamella does not configure into a shelf-like extension but rather, maintains a well-confirmed tight course around the columella, carrying the pronounced parietal continuation of this course all the way to the outermost extension of the aperture (Fig. 10E). This robust continuation of the columellar lamella forms a thick ridge extending as far as and aligning directly above the umbilical notch of this species (when viewed from below). This external feature clearly distinguishes *Z. kupitzense* from the others.

The umbilical notch of *Z. isselianum* (Turjeva jama, neotype locality) (Fig. 8E) is less emphasized in the appearance of forceful twisting as in *Z. kupitzense*. This emphasized twisting dynamic is evident in the nano-CT images of *Z. kupitzense* (Fig. 10A–F). The umbilical perspective of *Zospeum* sp. (Konečka zijalka) (Fig. 8I–L) shows a deeply set umbilicus for this morphospecies. Although taken from a fragment of the umbilical region (Fig. 8I), this view is informative enough to indicate that this notch is deeper set than in the other allied species comprising this study. The columellar lamella maintains a similar, slight sinuous extension as in *Z. alpestre* (Fig. 10G).

The deep interconnected pits forming continuous rows of wavy pattern on the upper teleoconch are markedly more pronounced in topotypic *Z. isselianum* (Turjeva jama) (Fig. 9A–F) than in *Zospeum* sp. (Konečka zijalka) (Fig. 9G–H). The interconnected shallow pitted rows on the teleoconch of *Z. amoenum* (Fig. 9M) are less pronounced and rather intermediary and moderate compared to the clear and well-defined topography of the rows in topotypic *Z. isselianum* (Turjeva jama) (Fig. 9A–F) and the deeply pitted waves of *Z. kupitzense* (Fig. 9I–L). However, the pitting on the protoconch of *Z. amoenum* (Ihanščica cave) is well pronounced though not distinctive to this species. Although pitting was well defined on the protoconch of all species in

this study, *Z. isselianum* (Turjeva jama) shows a distinctive pattern of densely set pits interspersed by bands of empty space and then more rows (Fig. 9D–E). Rheomorphic stretching is frequently encountered on the protoconch of *Z. isselianum* (Fig. 9A, D).

Discussion

Our molecular delimitation results singularly imply that:

- a) *Zospeum isselianum* (L1) is validated by molecular data from the area of the new precise type locality (Turjeva jama, Robič, Kobarid, Slovenia).
- b) *Zospeum* sp. (Konečka zijalka) (L2) is an unknown, highly morphologically variable *Zospeum* lineage (comprising specimens of *Z. isselianum* and *Z. cf. amoenum*) with ambiguous identity.
- c) Polytypy within the species *Z. alpestre* (i.e. *Z. alpestre alpestre*, *Zospeum alpestre kupitzense* and *Zospeum alpestre bolei*) needs reassessment.

Our studies of *Zospeum* shell morphology of the allied Eastern Alpine species show the wide degree of external shell variability and evidence of convergent evolution within these taxa. These investigations corroborate the findings of Bole (1974), Maier (1982, unpublished data), Slapnik (1991) and De Mattia (2003) in that *Zospeum* shells typically tend to be very variable in shape from one population to the other and frequently show wide variability within the same population. The most reliable conchological character, however, in differentiating between morphospecies in this study is the form and configuration of the columella. The columellar lamella on the other hand, is subject more to phenotypic variability in the degree of shell material invested in the composition of the lamella and the subsequent expression of planar extension. The external superficial texture of the shell was found to be differentiable in *Z. isselianum* and *Z. kupitzense* in this study whereby the pattern of microstructural pits on the shells of *Z. isselianum* and *Z. kupitzense* are specific for these two species.

The age of the shell, superficial encrustations, degree of preservation/freshness, and the degree of biomineralization affecting shell hardness vary, and thus, generally influenced moderate forms of superficial shell texture.

Zospeum shows characteristic pulmonate design with ellobioid affinity. The gross anatomy of *Zospeum* (Konečka zijalka) does not deviate significantly from other investigated *Zospeum* species (Giusti 1975, Maier 1982, unpublished data, Dörge 2010, unpublished data). The *Zospeum* individual in Dörge's (2010, unpublished data) cursory histological study was collected at the same time and came from the same population as the specimen presented here. Some structures such as statocysts in conjunction with the pedal ganglion were recorded as was a large albumen gland, suggesting that Dörge's (2010, unpublished data) specimen was likely collected in the female phase. In the Ellobioidea, the penial structure shows varying degrees of complexity (Martins 1998). In anatomical studies conducted on *Zospeum* so far, individuals in the male phase predomi-

nated (Giusti 1975, Maier 1982). The albumen gland was not detected in our study and may not yet have developed or may have been reduced on account of the male phase. The individual used in this study shows a conical, well-retracted penis into the body cavity, indicating the animal was collected during the male phase but not sexually active (Duncan 1975, Gómez 2001). As in most ellobioid genera, excepting for *Blauneria* Shuttleworth (Ellobiinae) and *Myosotella* Montserato (Pythiinae) (Barker 2001), the long vas deferens of *Zospeum* is embedded in the body wall and courses parallel to, but, independent of the penis. Maier's (1982, unpublished data) investigation of *Z. spelaenum schmidtii* shows a likely more mature state in size and elongated form as well as an apparent separation of the penial sheath from the penis versus the compact form and sheerness of the penial sheath in *Zospeum* (Konečka zijalka) here. The specimen in Maier's study was likely sexually active. In *Z. tellinii* (= *Z. spelaenum costatum* (Freyer, 1855)), Giusti (1975) described the penis as being extended and long as well as positioned outside the body parallel to the right tentacle. Our specimen (and that of Dörge (2010, unpublished data)) was collected in August and likely reflects seasonal protandry in *Zospeum*.

The radulae of *Zospeum isselianum* (Turjeva jama) here and *Zospeum* sp. (Konečka zijalka) (Dörge 2010, unpublished data; Jochum 2011) show a long median groove on some of the lateral teeth, which is a new structural feature for the "Carychium-type" radula defined by Martins (2007) for the Carychiidae. Diverse considerations along a second line of this study involve ecological aspects such as the relationship between tooth cusps and substrates, producing adaptive moderations of morphological detail. Mechanical factors such as forces acting on teeth during feeding produce adaptive solutions to pressure, such as development of compressional ridges (Hickman 1980). Moreover, the cusp of the tooth interacts with the substrate during feeding and is vulnerable to substrate characteristics and dietary preferences (Hickman 1980; Luchtel et al. 1997). Consequently, if tooth cusps are to most effectively function with the fine nuances of substrate composition and configuration (i.e. "grain"), superimposed elaborations likely evolve to correlate with the substrate grain. The grooves on the radulae of these two *Zospeum* here may well reflect such ecologically induced adaptive factors.

Based on the most unambiguous shell character found in this study, namely, the configuration of the columella and independently, the relationship of the columellar lamella with it, the bigger morphological assessment-vs-molecular delimitation picture can be broken down as follows:

- Columella slender, twisted; secondary columellar dilatation at base of columella; columellar lamella inclinate, extended. Lineage 1: *Zospeum isselianum* from new type locality.
- Columella robust, cylindrical, smooth; no columellar lamella: *Z. amoenum* (Potočka zijalka).
- Columella robust, hint of secondary dilation at base, smooth; reduced, un-elaborated (i.e. non-extended) columellar lamella. Lineage 2: *Zospeum* sp. (Konečka zijalka) appears to be morphologically an intermediary form of *Z. amoenum* and *Z. isselianum* morphotypes.

- Columella broadly clavate; columellar lamella thin and finely sinuous, extending to a short horizontal plane within the penultimate whorl. Lineage 3: *Z. alpestre* (Kamniška jama).
- Columella slender, twisted; slight secondary columellar dilation at base of columella; columellar lamella only similar to *Z. alpestre* but not the columella; pronounced parietal lamella formed from the inner track of the columellar lamella; parietal lamella remarkably long, extending to middle of shell above the umbilicus (bottom perspective). Lineage 4: *Z. kupitzense*.

This morphological analysis, in conjunction with the molecular results, suggests that lineage 2 *Zospeum* sp. (Konečka zijalka) possesses an intermediary columellar form and shows morphological affinity to both *Z. amoenum* and *Z. isselianum*. We cannot designate species status for this material at this time. On the one hand, these individuals are not *Z. isselianum* and can thus, be excluded as this species by our study here. On the other hand, *Z. amoenum* cannot be excluded because we did not investigate type-material of this species. This means the examination of L2 should be the focus of another investigation, incorporating topotypic material of *Z. amoenum* (locus typicus: Velika Pasjica, Gornji Ig, Slovenia) to clarify this issue. Interestingly, this cave is also the type locality of *Zospeum spelaenum schmidtii*. In regards to the bigger picture here, it is remarkable that Bole (1974) recorded three species of *Zospeum* from Ihanščica cave (*Z. alpestre isselianum*, *Z. spelaenum schmidtii* and *Z. amoenum*) while the Kuščer collection in Trieste houses another specimen of *Z. spelaenum* (Rossmässler, 1839) (Kuščer collection nr. 1186) from this very cave (figs 19–21, De Mattia 2005). In addition, this same cave was the origin of a specimen identified by Kuščer (*Z. schmidti striatum* nov. sp., NHMW Edlauer #48.657), which is currently recognized as *Z. spelaenum schmidtii*. In our study, specimens of *Z. cf. amoenum* (Fig. 9M–O) and *Z. isselianum* (Fig. 10H,I,L) also came from this cave. In context, we are likely witnessing Ihanščica cave as the site of a “species flock” whereby this monophyletic, relatively species-rich (for *Zospeum* at least), narrowly endemic group of closely related taxa all inhabit the same subterranean ecosystem (Bickford et al. 2006). On the other hand, Ihanščica cave and Konečka zijalka are ca. 40 km apart, noncontiguous and share no common river drainage systems where the *Z. isselianum* populations comprising this study could come in contact. Further investigation of *Zospeum* sp. (Konečka zijalka) may likely show a similarly complicated dynamic involving a very high regional/within-cave morphological variability producing many distinct morphotypes. Thus, the characterisation of several *Zospeum* morphospecies within Ihanščica cave could also be seen as a taxonomic over-interpretation of the intraspecific variability of a single species or a few species.

Based on the configuration of the columella plus the independent configuration of the columellar lamella, morphological differentiation of these lineages was generally possible. Although aperture dimensions and the expression of the columellar lamella in the aperture were highly rated in earlier assessments of *Zospeum* (Bole 1974), the value of these characters has been exaggerated. Since the columella functions as a form of scaffolding for the shell, it must conform to the internal needs and static requirements

of the animal. The size of the aperture is more a reflection of the size of the foot. More important however, is the mechanization of the columellar muscle during mobility, during the protraction of the penial organ and the role of the columellar muscle in radular function as these aspects largely have much to do with the configuration of the columella and the utilization of the columellar lamella for these specific processes.

Conclusions

Four evolutionary lineages of Eastern Alpine *Zospeum* were clearly molecularly delineated and assessed using various non-molecular methods. Based on differentiating shell features and the molecular species delimitation, a neotype for *Zospeum isselianum* was described. This study performed the first, non-destructive 3D-reconstruction using X-ray tomography (nano-CT) to differentiate shells of conchologically and geographically allied species of *Zospeum*. Since external shell morphology is too variable, specific focus was given to internal characters such as to the configuration of the columella and the orientation of the columellar lamella within the ovate conical shell. The columella was found to be the most reliable conchological character differentiating species of Eastern Alpine *Zospeum* taxa. SEM assessment infers that a median groove incised in the middle cusp of the lateral teeth of *Z. isselianum* (Turjeva jama, neotype locality) here and *Zospeum* sp. (Konečka zijalka) of previous investigations (Dörge 2010, unpublished data, Jochum 2011) reflects a phenotypic modification in intrinsic design. Seasonal proandry is inferred for *Zospeum*. New access to new means of interpretation has provided a refreshing taxonomic perspective for these microscopic ellobioid snails. Considering the results of Weigand et al. (2013) and our conchological investigations here, we find no reason to maintain the two subspecies entities currently comprising the *Z. alpestre* complex of the Eastern Alpine region. This study reduces the volume of previous *Z. alpestre*-laden eras by narrowing the status to two valid species: *Z. kupitzense* and *Z. alpestre* for this region. On the other hand, *Zospeum* sp. (Konečka zijalka) (L2) presents a new dilemma we didn't have before, namely, an intermediate form of *Z. cf. amoenum* and *Z. isselianum* in an morphologically amalgamated, *Z. amoenum*–*Z. isselianum* lineage. This incidence of character amalgamation likely reflects a condition involving a species flock and potential incipient speciation here. However, other plausible causes could involve hybridization or the use of a single gene fragment for delimiting species. Future investigations should focus on clarification of *Zospeum* sp. (Konečka zijalka) by including material of *Zospeum amoenum* collected at its type locality (Velika Pasjica, Gornji Ig, Slovenia). Our study lays the groundwork for future taxonomic investigations of *Zospeum*. We emphasize the value of applying a multifaceted, taxonomic approach to assess the frequent instances of intraspecific variability in other groups of highly morphologically variable species and not only for Southeastern Alpine *Zospeum* taxa (Schniebs et al. 2011, Puzin et al. 2014). Lastly, we hope this investigation presents valuable insights for future biodiversity studies of subterranean taxa as well as bears potential for influencing conservation management strategies within the Southern Alpine subterranean realm.

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Supplementary material I

List of localities of *Z. isselianum*

Authors: Adrienne Jochum, Rajko Slapnik, Annette Klussmann-Kolb, Barna Páll-Gergely, Marian Kampschulte, Gunhild Martels, Marko Vrabec, Claudia Nesselhauf, Alexander M. Weigand

Data type: occurrence

Explanation note: List of localities of *Z. isselianum*. A – Austria, BiH – Bosnia and Herzegovina, Cro – Croatia, Slo – Slovenia, Ita – Italy, CBSS – Collection of Croatian Biospeleological Society, CSR SASA – Malacological collection of the Biological Institute of the Centre for Scientific Research of the Slovenian Academy of Sciences and Arts in Ljubljana, SMNH – Malacological collection of the Slovenian Museum of Natural History, LIT – data from literature.

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